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SUPPLEMENTUM I

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Increased Brain Uptake of Mercury Caused by 2,3-Dimercaptopropanol (BAL) in Mice given Mercuric Chloride

By

M Berlin and T Lewander

(Received July 13 1964)

Most text-books of pharmacology recommend 2,3-dimercaptopropanol (dimercaprolum, Ph. Nord. BAL) as an antidote to mercury in acute poisoning, a use supported by both clinical experience and experimental findings (GILMAN *et al.* 1946 LONGCOPE *et al* 1946) There is, however no certain proof that BAL is effective in chronic mercury poisoning. FITZSIMMONS & KOZELKA (1950) in a brief report, stated that, though BAL decreases the mercury concentration in the kidneys of the rat and monkey it increases the mercury content of other organs. They did not find any increase in urinary mercury excretion on the contrary there was less mercury excreted than in untreated controls. ADAM (1951) also found that in the rabbit BAL caused a considerable decrease in the amount of mercury in the kidneys, but with markedly elevated excretion of mercury in the urine.

In a previous report (BERLIN & ULLBERG 1963a) it was shown that there is a considerable risk of mercury accumulation in the mouse brain from prolonged exposure It was therefore of interest to study the effect of BAL on mercury accumulation in the mouse brain, especially since BAL has been found to increase the mercury content of the brain after exposure to arylmercury (BERLIN & ULLBERG 1963b). In the study reported here this was done by comparing autoradiograms of sagittal whole-body sections of mice given BAL and radioactively labelled mercuric chloride with those of mice given only mercuric chloride In the second part of the work, two groups of mice were exposed to repeated daily doses of mercuric chloride one group was also given BAL. The total body retention of mercury was measured daily and after 16 days the animals were killed and the retentions in several of the organs were assayed.

Methods

Single exposure

Five pregnant CBA mice, obtained from the Genetic Institution of the University of Stockholm, were each given in the tail vein 0.5 mg mercury/kg body weight as $^{203}HgCl_2$ having a specific activity of 2 mC/mg and an equivalent amount of BAL, 0.3 mg/kg body weight. The mice were killed under ether anaesthesia at 1 hour 1 day 4 8 or 16 days after the injection by immersion in a mixture of acetone and solid carbon dioxide (−80°) Five pregnant mice of a control group were given the same dose of mercuric chloride and killed at the same intervals. Injections in both groups were given so that they were killed on a day near term. Sagittal whole-body sections, 20 μ thick, were cut by the method of ULLBERG (1958). Ten sections were chosen to represent most organs and each were autoradiographed together with a strip of film impregnated with stepwise dilutions of the injected mercurial to serve as a reference standard. The order of isotope concentration in the organs was then determined by a method previously described (BERLIN & ULLBERG 1963b) in which the darkening due to mercury in the tissue was compared by optical densitometry with the density of the reference standards.

Prolonged exposure

Thirty male CBA mice were divided into two equal groups so that the body-weight distribution was the same in both groups. All mice were subcutaneously injected daily for 16 days with a solution of $^{203}HgCl_2$ (specific activity of 50 μC/mg) at a dose corresponding to 1 mg mercury/kg body weight. One group also received daily subcutaneous injections of BAL, 2 mg/kg body weight, dissolved in peanut oil. The mice were housed in three cages, 10 to a cage, 5 from each group. The body load of

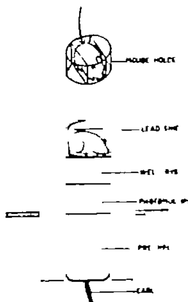


Fig. 1 Schematic diagram f apparatus used for making whole-body scintillation counts of mice. The mouse holder w s constructed of perspex.

mercury was determined by daily measurement in a body counter containing a scintillation detector with a well crystal and shielded by lead 5 cm thick (fig. 1). The mouse was placed above the crystal in a special container (fig. 1). The efficiency of this method of body counting was estimated by measuring a known amount of radioactivity injected into a mouse in the body counter and the same amount directly in the well crystal. The body count was regularly $\frac{1}{10}$ of the direct count in 5 trials. The animals were killed after 16 days exposure. The brain, kidneys, testes and liver were removed and dissolved in concentrated hydrochloric acid. The solutions so obtained were measured twice in the well crystal, 10,000 counts each time. The background was about 700 counts/min., and no value measured fell below 20 % of that.

Results

Autoradiographic findings after single exposure

A comparison between the autoradiograms from the BAL treated animals and the controls reveals that BAL changes the mercury distribution. A difference is already seen one day after the injection (fig. 2), but can not be detected after 4 to 16 days. Although the whole-body autoradiograms do not permit any definite conclusion about the precise nature of the observed difference in mercury distribution, one main effect is that mercury is kept in the blood for a longer time in the BAL injected animals. If the density of the blood is taken as standard in Figure 2, the liver and kidneys of the animals given BAL clearly contain relatively less mercury than those of the control animals. In the subsequent autoradiograms, at 4, 8 and 16 days after injection, no clear difference in distribution was observed between treated and control animals. No change in the degree of mercury penetration into the foetus was observed after BAL injection.

Whole-body counts and organ assay after repeated exposure

There were no differences observed between BAL-treated animals and controls during the period studied in body accumulation of mercury as determined by whole-body counting. *Figure 3a depicts the daily body burden of mercury in each control animal and the group mean, fig. 3b* does the same for the BAL-treated animals.

The measurement of the radioactivity retained in the organs at the end of 16 days did not reveal any statistically significant differences between the two groups for the mercury contents of testes, kidneys or liver (*Table 1*). However, there was about 20 % more mercury in the brains of BAL treated animals than in those of the controls. This difference is significant ($0.001 < p < 0.01$).

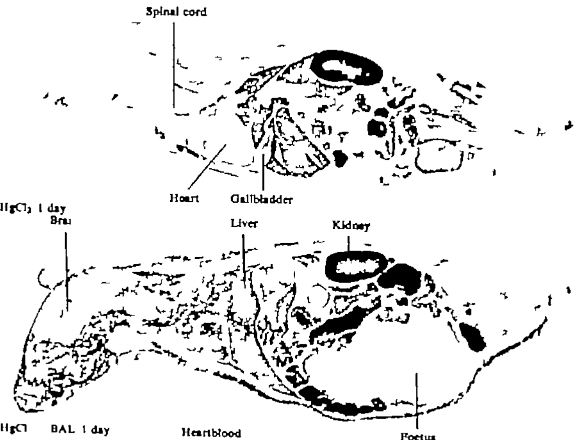


Fig. 2. Autoradiograms of sagittal whole-body section of mice 1 day after injection of $^{203}HgCl_2$ (upper) and $^{203}HgCl$ + BAL (lower). Dosages in text. The lower section is from a pregnant mouse.

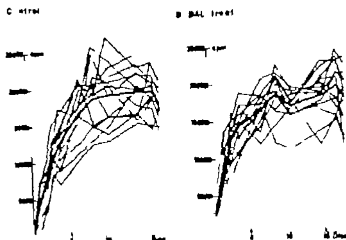


Fig. 3 Consecutive total body scintillation counts of mice (A) after $^{203}HgCl_2$ (B) after $^{203}HgCl_2$ + BAL. Each thin line represents values for a single animal the heavy line represents the group mean.

Table I

^{203}Hg retained after 16 days in some organs of 15 BAL-treated animals and 15 controls.

		BAL + Hg		Hg	
		Mean	Range	Mean	Range
Brain	Organ weight (g)	0.415	0.384–0.433	0.418	0.392–0.434
	Activity (cpm)	292	174–360	226	100–326
	cpm/g	703 ± 36	420–764	585 ± 26	423–756
Testes	Organ weight (g)	0.192	0.117–0.212	0.191	0.157–0.210
	Activity (cpm)	772	328–1413	687	312–885
	cpm/g	4130 ± 456	2660–7444	3596 ± 163	2547–4785
Liver	Organ weight (g)	1.074	0.781 1.374	1.081	0.791 1.310
	Activity (cpm)	12684	6658–19780	12004	7585–19068
	cpm/g	12534 ± 1421	5326–20231	11500 ± 1384	7102–24106
Kidney	Organ weight (g)	0.412	0.326–0.504	0.398	0.346–0.440
	Activity (cpm)	40194	29914–49545	43173	30073–56179
	cpm/g	99920 ± 5970	67030–1 4174	107154 ± 5068	78168–145541

Discussion

The autoradiograms show that BAL given with a single dose of mercuric chloride causes a redistribution of mercury in the body The most probable explanation of this is that BAL forms a stable complex with mercury the distribution of which differs from that of inorganic mercury

The observed differences are consistent with the findings of ADAM (1951) in the rabbit and FITZSIMMONS & KOZELKA (1950) in the rat and monkey To be especially noted is the effect of BAL on the mercury content of the kidney The decrease of mercury in renal tissue found by these workers, and clearly seen in our 1 hour and 1-day autoradiograms, explains the favorable effect of BAL treatment in acute mercury poisoning in which kidney damage is the chief danger to life. An interesting question is where the mercury mobilized by BAL goes when it leaves the kidney ADAM found an increase in urinary mercury excretion in the rabbit FITZSIMMONS & KOZELKA found a decrease in the rat. An increased excretion of mercury in the urine has also been observed in clinical material Since there was in BAL treated mice repeatedly exposed to mercury no decrease in the body load of mercury it may be concluded that, if BAL increases the urinary excretion of mercury it does so at the expense of mercury excreted by other routes, perhaps in the bile or through the intestinal wall

It has previously been shown (BERLIN 1963) that in the rabbit, about 50 / of the mercury infused during a 4-hour period was accumulated in the kidneys. It is also likely that, in the mouse, about half the body load is in the kidneys In order to be detected by the autoradiographic techniques used in this study the decrease in renal concentration induced by BAL has probably to be of the order of 1 2. This means that about ¼ of the body load was distributed elsewhere in the BAL-treated mice On the other hand, in the study of prolonged exposure, no significant differences in mercury content were observed among the organs studied other than in the brain. This may however be explained by the rapid detoxification and elimination of BAL demonstrated by TAMBOLINE *et al* (1955) If the injected BAL combines with mercury in the body and effects a change in its distribution, subsequent breakdown of this complex may leave the mercury accordingly redistributed. The results of TAMBOLINE *et al* suggest that most of the BAL is excreted after 48 hours. If this is correct, it follows that measurements of organ concentration obtained in the repeated exposure experiment will show no more than the effect of the last BAL injections. The daily BAL dose in this experiment was rather small compared with the total load of mercury accumulated by the end of the experiment and this could explain why significant differences in mercury content were found only in one of the organs studied The brain in BAL-treated animals showed a considerable increase in mercury concentration In previous experiments (BERLIN & ULLBERG 1963b) it was shown that the uptake of mercury in the brain after a single dose occurs slowly over a period of several days. The mechanism of the brain uptake of mercury is not known therefore it is difficult to explain why the mercury content in the brain is increased by BAL. Either the BAL-mercury complex penetrates the blood-brain

barrier more readily than mercury itself or the redistribution of mercury in the body makes more mercury available for transport to and uptake by the brain.

Summary

The effect of dimercaptopropanol (BAL) (0.3 mg/kg) on the body distribution of mercury in mice given a single dose of $^{203}HgCl_2$ (0.5 mg Hg/kg) was studied by autoradiography of sagittal whole-body sections. It has been clearly shown that BAL effects a redistribution of the body mercury load, including a diminution in renal concentration. This has been taken to indicate the existence in the body of a BAL-mercury complex.

Mice in a second series were given daily injections of $^{203}HgCl_2$ (1.0 mg Hg/kg) for 16 days half also received 2 mg BAL/kg daily. Daily whole body scintillation counts revealed no increase in mercury elimination from the BAL-treated animals. However organ counts showed that at the end of 16 days 20 / more mercury had been accumulated in the brains of BAL-treated mice.

Acknowledgement

A grant from the Swedish Medical Research Council is gratefully acknowledged.

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Apparent Depression in the Absorption of Strychnine, Alcohol and Sulphanilamide after Oral Administration of Sodium Fluoride, Sodium Oxalate, Tetracemin* and Sodium Phytate

By

Erling Sögnen

(Received August 3 1964)

Lethal doses of chloralose and barbiturates or doses that normally produce deep unconsciousness, have a far less toxic effect when administered together with calcium binding substances (Ca b.s.). The plasma barbiturate level was found to be lower during the first few hours after oral administration when the barbiturate was given along with Ca-b.s. than when given alone.

A lowered plasma level is only obtainable by oral administration of Ca-b s together with the barbiturate. Hence there is reason to suppose that the absorption from the intestinal tract is delayed (SÖGNEN 1961).

The work here reported has been carried out to investigate whether Ca b.s. modify the effects or lower the plasma levels of other substances differing in chemical composition and pharmacological action.

Strychnine nitrate was chosen as a representative of the alkaloids, because besides having a prompt action, it yields plain readily observable signs of poisoning.

BERGOREN & GOLDBERG (1940) have shown that ethanol is transferred passively from the intestinal lumen by simple diffusion. This was therefore chosen as a representative of the substances known to have this particular property

Sulphanilamide was chosen because it is supposed to have little effect in itself on the factors influencing absorption and in general on other functions of the organism.

Moreover suplhanilamide is soluble in an acid as well as an alkaline environment and, at the concentrations in question, also in a neutral

* Tetracemimum (NFN) Edetic acid (BAN) Edathamil (NNR)

environment at 37° Its physicochemical properties (pK_a 10.4) cause its absorption rate to be affected but little by minor variations of pH in the intestinal tract (SCHANKER *et al* 1958)

Material and Methods

For most of the experiments male albino rats weighing from 160 to 290 g were used. The animals were not fed, but had access to water during the night preceeding the experiment When nothing to the contrary is stated, the solutions given were administered by a stomach tube of glass fastened to a record syringe. Weighing of the animals, fixing them in position and administration, carried out by passing the glass tube into the stomach, hardly ever took more than one minute.

In the strychnine experiments we took care not to touch the animals or excite them by sounds or abrupt movements after the administration.

For some of the sulphanilamide experiments we used rabbits. To avoid washing the rubber tube as had been done in previous experiments, a plastic tube (3 mm in diameter) was passed through this.

After introduction into the stomach, the tip of the plastic tube was passed about 2 cm further down than the rubber tube. The sulphanilamide solution was administered by a record syringe. The plastic tube had been filled in advance with the same solution. By this procedure we also avoided such irregular infusion periods as air block and obstructions may cause when using an ordinary rubber tube. Use of funnel and graduated cylinder was likewise avoided, so that the rabbits could easily be manipulated with without assistance. The rabbits were fasted for 48 hours before the experiments, but had access to water

Blood samples for alcohol determination were drawn by cardiac puncture and transferred to special Widmark ampoules immediately after sampling.

The samples for sulphanilamide determination were drawn by cardiac puncture or from the tip of the tail after transection with sharp scissors. The rabbits blood samples were withdrawn from the ear vein, as in the experiments reported in a previous paper (SÖGNEN 1961).

Solutions. Strychnine nitrate 0.10 and 0.15 % w/v in aqueous solution ethanol 16 % sulphanilamide 1 % w/v in 0.9 % sodium chloride containing 1 mg/ml glucose were all prepared immediately before use and administered at approximately body temperature. In the control experiments Ca-b.s. was replaced by sodium chloride in the test solutions. Neutralised tetracemin was used.

Sodium phytate was prepared from wheat bran, as described by POSTERNACK (1921). The phytic acid is extracted with hydrochloric acid and precipitated with ferric chloride the ferric phytate is dissolved in sodium hydroxide, and the ferric hydroxide is filtered off. Sodium phytate is precipitated with alcohol, redissolved repeatedly in water and left to crystallize. Phytin phosphorus in the final product was determined as described by HEUBNER & STADTLER (1914). The phytate concentrate contained 6.83 phytin phosphorus, an amount in fair agreement with that found by other workers in concentrates (PEDERSEN 1940).

Analytical methods

The alcohol analyses were performed according to Widmark. Heparin was used as anticoagulant at the sampling. The contents of reducing constituents of the heparin were found to have no influence on the results.

For the sulphanilamide analyses we employed KING *et al.*'s (1942) modification of BRATTON & MARSHALL's method (1939). The total sulphanilamide concentration was estimated after boiling in narrow test tubes graduated at the 5 ml level. No perceptible evaporation took place during the boiling, and adjustment of the volume after addition of the reagents was practically never required.

To facilitate the mixing of hydrochloric acid and the other reagents, glass rods were used, which were left in the test tubes during the boiling and also throughout the procedure of the remaining analysis. The colour appearing after addition of *N-(1 naphthyl)-ethylenediamine* was very photosensitive. Exploratory experiments showed that at low sulphanilamide concentrations the extinction values dropped about 50% in 15 min. if the test tubes were exposed to sunlight, whereas the colour remained unchanged in tubes left in the dark. The measurements were carried out on a Beckmann spectrophotometer DU about 15 min. after the reagents had been added.

Results

Strychnine nitrate

In exploratory experiments 5 mg strychnine nitrate /kg body weight proved fatal to one rat out of five. After 10 mg/kg four rats out of five died The animals died 35 minutes to 2 hours after oral administration

Clearly lethal were 15 mg/kg. In a group of ten rats all the animals receiving thus displayed convulsive fits after 9 to 28 minutes and died 11 to 34 minutes afterwards.

To ten rats 15 mg strychnine nitrate/kg were given together with 50 mg sodium fluoride/kg. None of the animals died or had convulsions.

The same dose of strychnine was also administered together with 400 mg tetracemin/kg to five rats and together with 150 mg sodium oxalate/kg to five others. None of these rats had convulsive fits or died

These experiments showed that Ca-b.s. in the amounts mentioned raised the tolerance to oral doses of strychnine nitrate given together with and in the same solution

Table 1

Effects of calcium-binding substances the blood alcohol concentration 1 hour after oral administration of alcohol 1 6 g/kg in 16% (w/v) solution.

Ca-b.s. mg/kg	Number of animals	Blood alcohol concentration in %		Per cent of control
		Mean	Range	
Sodium fluoride 50	8	0.31	0.04–0.48	34.8
Tetracemi 400	2	[illegible]	0.16–0.29	24.7
Sodium oxalate 150		0.30	0.40–0.10	33 7
Controls	8	0.89	0.57–1.24	—

Alcohol

As shown in table 1 blood samples withdrawn one hour after administration showed that sodium fluoride, sodium oxalate and tetracemin lowered blood concentration of alcohol appreciably

Sulphanilamide

Table 2 illustrates a comparison between the concentrations of sulphanilamide in blood samples from non-anaesthetized and anaesthetized rats in the presence or absence of Ca-b.s. during the initial phase of the absorption. The anaesthetized rats were given 1250 mg/kg of a 12.5 / solution of urethane intraperitoneally 15 min. before intubation. During the experiments the animals lay on a heat-regulated table kept at a temperature of 38°

Table 2

Effect of tetracemin on the sulphanilamide concentration in anaesthetized and non-anaesthetized rats during the initial period of absorption.

Blood concentration of sulphanilamide (µg/ml). (Each figure mean value for 5 new animals)

	Anaesthetized	Non-anaesthetized
Sulphanilamide 100 mg/kg orally		
10 min.	34	57
20 min.	37	70
Sulphanilamide 100 mg/kg + Tetracemin 372 mg/kg orally		
10 min.	26	33
20 min.	30	34

It is seen that the effect of Ca-b s. on sulphanilamide concentration was small in the anaesthetized compared with the non-anaesthetized rats.

The table also shows that the action of Ca-b.s. in depressing absorption was pronounced after 10 min. and that the concentration obtained at this time in the experimental group altered little within the first hour (fig. 2) Fig 1 a, b & c and fig. 2 illustrate the effects of Ca-b.s. on the blood level of sulphanilamide from 1 to 12 hours after administration to rats. Equimolar amounts of Ca-b.s. were here given to the animals in the experimental groups, that is 42 mg sodium fluoride, 134 mg sodium oxalate and 1000 mg sodium phytate/kg body weight, as well as neutralized tetracemin corresponding to 372 mg/kg of the disodiumsalt of tetracemin. Sulphanilamide at 1 / concentration was dissolved in an 0.1

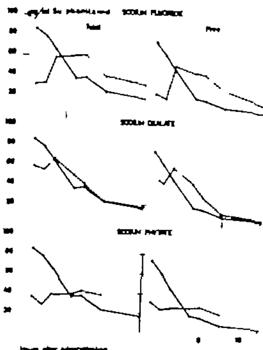


Fig. 1 a, b and c. Experimental group ●———● Control group ○————○ Effects of equimolar amounts of calcium-binding substances on the blood concentration of sulphanilamide in rats after sulphanilamide 100 mg/kg by stomach-tube

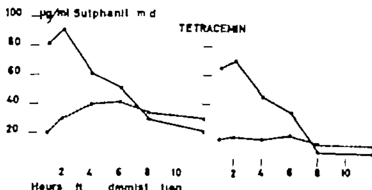


Fig. 2. Experimental group ●———● Control group ○————○ (calcium tetracemin). Effects of tetracem 37 mg/kg on the blood concentration of sulphanilamide in rats after sulphanilamide 100 mg/kg by stomach-tube.

molar solution of these substances The sodium phytate being of an own production, the amount mentioned was only approximately equimolar with the other Ca b.s The molecular weight of anhydrous sodium phytate is 924 *The solutions also had sodium chloride* and glucose added, as indicated above

The control group common to fig. 1 a, b & c, was given a solution osmotically equivalent to the test solutions containing sodium chloride. The control group in fig. 2 received calcium tetracemin in amounts equimolar with the tetracemin in the experimental group

Each point on the curves represents the mean of the blood sulphanilamide concentrations in two rats. As a rule there were only small differences in concentration at any one time.

In the experiments on rats (figs. 1 & 2) tetracemin and sodium phytate proved to be the most active depressors of absorption No particular importance should be attached to the quantitative differences in the experimental groups. Oxalate was generally seen to have the least depressing action.

Fig. 3 a, b & c illustrate the results of similar experiments on rabbits. The curves in fig. 3 a & c represent the blood levels in one control and one experimental animal. In fig. 3 b the curve is based on the means of the sulphanilamide concentrations in two controls and two experimental animals. The dosage and solution were the same as in the rat experiments.

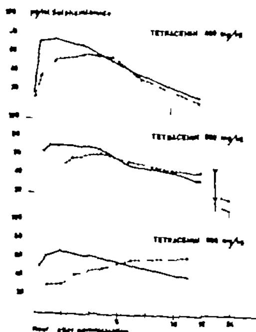


Fig. 3 Experimental group ● ——● Control group O———O Effects of different doses of tetracemin on the blood concentration of sulphanilamide in rabbits after sulphanilamide 100 mg/kg by stomach-tube.

The chart also shows that the greatest depressing action of tetracemin was obtained with a dose of 400 mg/kg, a dose within the same range as that in the rat experiments (372 mg/kg)

The sulphanilamide concentration reached maximum at a later time with a tetracemin dose of 800 mg/kg than after smaller doses. The curves for the blood sulphanilamide concentrations run practically identical courses after 400 or 600 mg tetracemin/kg.

Table 3 records the results of premedication of Ca b.s at the same doses as reported above one week before the sulphanilamide administration

Table 3

Sulphanilamide bsorption in albino rats 7 days after pretreatment with single oral dose of calci m-binding substance. Dose of sulphanilamide 100 mg/kg in 1 solution. Period of absorption 2 hours.

Pretreatment 1 ml/100 g	Number of nimals	Mean conc. of sulphanilamid in blood, µg/ml	Per cent of control	P >
Tetracemin 0.1 M	4	87 ± 6.6	93.6	0.05
Sodium oxalate 0 1 M	4	93 ± 11 9	102..	0 05
Sodium fluoride 0.1 M	4	98 ± 6.3	107 7	0.05
Control 0 9 Sodium chloride	4	91 11 6		

It is seen that the sulphanilamide concentration was unaffected in the experimental group.

Ca b.s. cause secretory alterations in the gastro-intestinal tract and might cause pH alterations in the intestine Though pH alterations are unlikely to influence sulphanilamide absorption, the experiments recorded in table 4 are carried out in order to observe the blood level of sulphanilamide administered in acid or alkaline solution

Table 4

Blood sulphanilamide concentratio in rats 1 hour fter oral administration of sulphanilamide 100 mg kg acid nd lk line solutions

Solution	Number of animal	Mean conc f ulpha ilamide µg ml	Difference
0 1N HCl	5	84 0 5 95	
0 1N-NaOH	5	80 8 9 94	p 0 05

Discussion

In intestinal absorption materials are transferred from the lumen of the gut across the intestinal mucous membrane to blood and lymph vessels (KORELITZ & JANOWITZ 1957) This process is called by some authors translocation (FISHER & PARSONS 1949) or transepithelial translocation from the gut lumen into the extracellular fluid (HOGBEN 1958)

In our investigation the estimation of intestinal absorption is limited to a determination of the concentrations of alcohol and sulphanilamide in the blood. The blood concentration of a substance given orally does however depend on other factors besides transepithelial translocation or absorption alone. Thus stomach emptying (REYNELL & SPRAY 1956) and motility (HOGBEN 1958) play a considerable part in determining the rate of exposure to more absorptive surfaces.

When the term "reduced intestinal absorption" is used in this paper it is to indicate that the blood concentration of the substance in question is reduced Whether permeability motility or other factors are affected will have to be determined by other methods

In the investigation Ca-b.s. were found to reduce the absorption rates of strychnine, alcohol and sulphanilamide The same retarded rate of absorption has been demonstrated when barbiturates were given along with Ca-b.s. (SÖGNEN 1961) Similarly the reduction in toxic effect of red squill in presence of Ca-b.s., demonstrated by DYBING DYBING & STORMORKEN (1952) has been suggested as most likely due to reduced intestinal absorption (DYBING & SÖGNEN 1958)

The action of Ca-b.s. in depressing absorption must be presumed non specific, that is, independent of the substance to be absorbed

Substances normally unabsorbable, e. g. heparin and heparinoid substances, however are absorbed in presence of tetracemin (WINDSOR & CRONHEIM 1961) The apparent discrepancy between this finding and those of our investigation has been discussed in a previous paper (SÖGNEN 1965).

The unmistakable protective action against a rapidly acting lethal dose of strychnine showed that the absorption-delaying action of Ca-b.s. manifests itself immediately after the administration. This was borne out by the results of the experiments with sulphanilamide (table 2) in which the absorption was seen already to be depressed after 10 minutes in non-anaesthetized animals.

In anaesthetized animals the lowering of the blood level was less clear and at any rate much less pronounced The anaesthesia as such seemed to cause a delay in absorption, which to some extent masked the effect of Ca-b.s. (table 2) (This observation will be returned to later)

As shown by TRAVELL (1948) the absorption of strychnine from the stomach depends on pH At a high pH strychnine will be somewhat dissociated and pass the *gastric mucosa more quickly than at a low pH* where it is practically not dissociated Thus, 5 mg strychnine sulphate in a solution at pH 8 injected into the stomach of an anaesthetized cat proved fatal within 24 minutes, whereas the same dose injected in a solution at pH 3 did not cause death.

With use of the stomach tube the absorption of strychnine from the stomach is probably insignificant compared with that from the small intestine. Some of the administered dose will pass into the small intestine immediately after administration and a considerable proportion will generally have left the stomach a few minutes after the intubation. (Shown in exploratory experiments with phenol red by SÖGNEN)

It is therefore unlikely that alterations in gastric pH could be the direct cause of the action of Ca b.s in depressing absorption. As, however the rate of transfer through the small intestine membrane also depends on the dissociation of the substance concerned (SCHANKER *et al* 1958), a change in pH might here well be thought to influence the absorption rate of strychnine Exploratory investigations showed, in fact, a marked tendency towards increased tolerance of strychnine infused directly into the small intestine in a solution at pH 3

The dissociation of sulphanilamide is practically unaffected by fluctuations in intestinal pH. As delayed absorption was clearly demonstrable with this drug, too an effect on dissociation can probably be excluded as the cause of depressed absorption.

The results set out in table 4 show that sulphanilamide administered in 0 1 N HCl and 0 1 N NaOH gave identical blood sulphanilamide concentrations after one hour Although the buffer capacity of the gastrointestinal secretions is efficient, we might expect some influence on the absorption rate if this depended to any extent on the pH of the solution.

Since alcohol transport, which is passive (BERGGREN & GOLDBERG *1940) was seen also to be delayed by Ca-b s., most likely it is not* an active transport mechanism that is influenced by Ca b.s in the intact animal.

Fig. 1 & 2, which illustrate the blood sulphanilamide concentrations in rats in the presence of Ca b.s., show that tetracemin and sodium fluoride lowered the blood level more than did sodium oxalate and sodium phytate. Expressed as percentages, the concentration of free sulphanil amide was lowered more than that of total sulphanilamide. There was, in other words, a higher degree of acetylation at the lower blood con centrations in the experimental groups.

The curves have been plotted on the basis of blood samples withdrawn

from two new individuals at different times after the administration. Each experimental group thus comprised 14 rats. To obtain parallels for the suphanilamide analysis, it would be necessary to draw not less than 0.5 ml of blood in each sample. Repeated withdrawals of such large samples from rats of this size with a blood volume of about 10 ml would soon alter the haematocrit values and cause disorders of distribution. Also, repeated cardiac puncture might cause circulatory disorders of cardiac origin. Repeated blood samplings from the tail likewise seemed to cause development of local circulatory disorders. The samples for analysis should therefore not be drawn till 0.2–0.3 ml of blood has dropped from the point of cutting the tail. By drawing samples from this site one thus "economizes" but little on the total blood volume.

The sulphanilamide experiments on rabbits (fig. 3 a, b & c) were also carried out with a view to comparing the blood level curve for diemal (SÖGNEN 1961), which is excreted slowly in the urine, with that for sulphanilamide, whose renal excretion is relatively fast. It is seen that the sulphanilamide concentration also remained higher in the experimental groups than in the control groups within the period 6 to 24 hours after administration.

Further the curves show that the absorption did not become increasingly depressed by raising the tetracemin dose, though after doses of 800 mg tetracemin/kg the blood level peak occurred later than after lower doses. No acute toxic side-effects were noticed after the largest doses of tetracemin.

Histological examinations of different small intestine segments taken from rats at different times after administration showed that Ca b.s. had caused no morphological changes demonstrable by the usual methods (ERICHSEN & SÖGNEN unpublished).

None of the animals in the experimental groups displayed signs of diarrhoea during a period of 12 hours after the experiments.

The absorption experiments on rats that had, one week before the sulphanilamide administration, been given Ca-b.s. at the same doses and concentrations as used in the other experiments showed that the effect of Ca-b.s. on the absorption of a sulphanilamide is reversible.

Summary

In the intact rat the toxic effect of strychnine is markedly decreased and the blood concentration of alcohol and sulphanilamide is reduced when the compounds are given in oral co-administration with the calcium binding substances, sodium oxalate, sodium phytate, sodium fluoride and tetracemin. As for sulphanilamide the results are verified in experi-

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The curves have been plotted on the basis of blood samples withdrawn

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Effects of Calcium-Binding Substances on Urine Flow and Renal Sulphanilamide Excretion

By

Erling Sögnen

(Received August 3 1964)

It has been shown that the plasma levels of barbiturates, sulphanilamide and alcohol are lowered, as compared with those in a control group when the drugs are given simultaneously with calcium binding substances (Ca-b.s.) by mouth (SÖGNEN 1961 & 1965 a).

In theory such lowering in plasma level could be due to increased excretion of the drugs in the urine. As stated in the discussion on the exploratory experiments with barbiturates, such a mechanism is fairly unlikely to exist here. As, however sodium fluoride is known to increase urine flow considerably (WADDEL 1884) it has been considered a matter of interest to study the effect of such increased urine flow on the excretion of sulphanilamide.

Ca b s. may be thought to have effects manifesting themselves in the urine flow They can diminish the urine flow by reducing the absorption of water from the intestinal tract (SÖGNEN 1965 c & e) They may also, depending on the extent of their absorption, possibly have an independent effect on renal function, which will affect the urine flow Changes in sulphanilamide excretion may conceivably be dependent on or independent of the urine flow

Material and Methods

Fasting male rats weighing from 200 to 300 g were used they had access to water until 2 hours before the experiments.

Groups of three animals each were placed in cages with wire floors arranged over a funnel system in which faeces and urine could be separated.

The urine was collected in graduated cylinders in which the urine volume was read directly The animals had no access to food or water during the experiments.

The animals were given a 1 % (w/v) solution of sulphanilamide in a 0 9 % sodium chloride solution containing 1 mg/ml glucose. Ca-b.s. were administered in this solution in further specified amounts. In the control solution the Ca-b.s. were compensated for by sodium chloride. The amount of fluid administered always constituted 1 % of the body weight and had a sulphanilamide concentration corresponding to 100 mg/kg. The solution had approximately body temperature when administered.

The total content of sulphanilamide in the urine was estimated as in preceding experiments (SÖGNEN 1965 a)

Results

Before the experiments the rats were allowed to drink water *ad lib.*, and they were not kept in rooms with heat and moisture regulation An Attempt was therefore made to clarify the question of accidental variations in urine flow

On six different days, groups of three rats each were placed in test cages for 24 hours. The average urine volume from these 18 animals was 0 843 ml/100 g (range 0 75–0.93 ml/100 g) After loading them with 1 ml/100 g of a 0 9 % sodium chloride solution containing 1 mg/ml glucose, the average urine volume after oral administration to 15 rats, in separate groups of three each, amounted to 1 8 ml/100 g (range 1.25–2.20 ml/100 g) After intraperitoneal administration, the urine volume for 15 animals averaged 1 4 ml/100 g (range 1.32–1 60 ml/100 g) There is thus a considerable variation in urine volume after oral water loading. In tables 1 to 6 the results of each experiment are therefore recorded by comparing the experimental and the control group for each day

Discussion

The mean values in tables 1 to 6 indicate the results of oral and intraperitoneal sulphanilamide administration and water loading under the influence of tetracemin, sodium oxalate and sodium fluoride, respectively

*Tetracemin**

The percentage recovered of sulphanilamide as well as the urine volume were found to be reduced when the sulphanilamide solution was given simultaneously with tetracemin by mouth (tables 1 & 4).

Intraperitoneal injection of the same solution affected neither sulphanilamide excretion nor urine volume.

* Tetracеminum (NFN) Edetic acid (BAN) Edathamil (NNR)

Table 1

Effect of tetracemin 186 mg/kg orally on sulphanilamide excretion and urine volume in rats given sulphanilamide 100 mg/kg in 1% solution (three animals in each group).

	% of sulphanilamide dose recovered in urine after 24 hours			Urine volume, ml/100 g		
Exp. no.	Control	Tetracemin	Ca-b.s./Control	Control	Tetracemin	Ca-b.s./Control
I	82.0	54.0	0.66	2.10	1.13	0.54
II	79.0	70.0	0.89	1.80	1.30	0.72
III	80.0	65.0	0.81	1.50	1.16	0.77
Mean	80.3	63.0	0.79	1.80	1.20	0.67

Table 2

Effect of sodium oxalate 67 mg/kg orally on sulphanilamide excretion and urine volume in rats given sulphanilamide 100 mg/kg in 1% solution (three animals in each group).

	% of sulphanilamide dose recovered in urine after 24 hours			Urine volume, ml/100 g		
Exp. no.	Control	Sodium oxalate	Ca-b.s./Control	Control	Sodium oxalate	Ca-b.s./Control
I	79.0	56.6	0.72	1.43	0.74	0.52
II	88.8	70.7	0.80	1.60	0.78	0.49
III	82.0	74.0	0.90	1.76	1.00	0.57
Mean	83.3	68.0	0.81	1.59	0.87	0.52

Table 3

Effects of sodium fluoride 1 mg/kg orally on sulphanilamide excretion and urine volume in rats given sulphanilamide 100 mg/kg in 1% solution (three animals in each group).

	% of sulphanilamide dose recovered in urine after 24 hours			Urine volume ml/100 g		
Exp. no.	Control	Sodium fluoride	Ca-b.s./Control	Control	Sodium fluoride	Ca-b.s./Control
I	78.0	41.0	0.53	1.60	2.95	1.84
II	83.0	67.0	0.81	1.12	4.97	4.44
Mean	80.5	54.0	0.67	1.36	3.96	3.14

Table 4

Effect of intraperitoneal tetracemin 186 mg/kg on sulphanilamide excretion and urine volume in rats given sulphanilamide 100 mg/kg in 1 % solution (three animals in each group)

Exp no	of sulphanilamide dose recovered in urine after 24 hours			Urine volume, ml 100 g		
	Control	Tetracemin	Ca-b.a. / Control	Control	Tetracemin	Ca b.a. / Control
I	79.4	78.7	0.99	1.84	1.80	0.98
II	75.1	80.0	1.07	1.73	1.78	10.2
III	82.7	78.0	0.94	1.68	1.98	1.18
IV	75.9	65.4	0.86	1.86	1.74	0.94
Mean	78.3	75.5	0.96	1.78	1.82	1.03

Table 5

Effect of intraperitoneal sodium oxalat 67 mg/kg o sulphanilamide excretion and urine volume in rats given sulphanilamide 100 mg/kg in 1% solution (three animals in each group).

Exp no.	/ of sulphanilamide dose recovered in urine after 24 hours			Urine volume, ml/100 g		
	Control	Sodium oxalate	Ca b.a. / Control	Control	Sodium oxalate	Ca-b.a. / Control
I	80.1	56.8	0.71	1.59	4.21	2.65
II	70.2	69.5	0.99	1.76	4.05	2.30
III	79.8	79.4	0.99	1.70	4.96	2.92
IV	81.0	64.1	0.79	1.21	3.06	2.53
Mean	77.8	67.1	0.87	1.57	4.07	2.60

Table 6

Effect f intraperit neal sodium fluoride 21 mg/kg on sulphanilamide excretion and urine volume in rats given sulphanilamide 100 mg/kg n 1% solution (three animals in each group)

Exp. no.	% f sulphanilamide dose recovered in urine after 24 hours			Urine volume, ml/100 g		
	Control	Sodium fluoride	Ca-b.a. / Control	Control	Sodium fluoride	Ca-b.a. / Control
I	87.1	59.2	0.68	2.23	3.03	1.36
II	79.2	74.8	0.94	2.04	3.99	1.95
III	72.2	48.1	0.67	1.67	4.65	2.78
IV	76.9	76.6	0.99	1.64	6.13	3.73
Mean	78.9	64.7	0.82	1.90	4.45	2.46

Sodium oxalate

Sodium oxalate given by mouth together with sulphanilamide had a depressing effect on sulphanilamide excretion and on urine flow (tables 2 & 5)

Intraperitoneal injection of the same solution increased the urine flow but did not increase sulphanilamide excretion

Of oxalate only so much is absorbed as is not precipitated by calcium in the intestinal tract (SCHMIDT NIELSEN & SCHMIDT NIELSEN 1944), and effects on the kidneys after oral administration of the doses employed can hardly be expected. This is supported by the fact that oxalate increased the secretion of urine only when given intraperitoneally The decrase of the urine flow after oral administration may be due to the reversal of gastro-intestinal water transport referred to above.

Sodium fluoride

The actions of sodium fluoride on the functions studied differ from those of sodium oxalate only by the increased volume of urine produced after oral administration (tables 3 & 6) Thus may be due to the fact that sodium fluoride is absorbed from the gastro-intestinal tract in a large measure (ZIPKIN & LIKINS 1957)

Conclusion When administered by mouth together with sulphanilamide, all the Ca b. s. reduced the amount of sulphanilamide recovered in the urine Ca b s. never contributed towards increasing the renal sulphanilamide excretion, even when the urine flow was considerably increased. The lowered plasma levels of sulphanilamide demonstrated previously (SÖÖNEN 1965 a) must therefore be due to factors other than increased renal secretion

Summary

In intact rats after oral administration of calcium-binding substances and sulphanilamide together renal excretion of the latter is reduced

After intraperitoneal administration of the same substances, renal excretion of sulphanilamide is not increased.

Increased urine flow did not cause an increase in sulphanilamide excretion

The lowered plasma concentration of sulphanilamide demonstrated in previous experiments must therefore be due to factors other than increased renal excretion.

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Effects of Calcium-Binding Substances on the Absorption of Fluid and Sulphanilamide from Isolated Intestinal Segments In Situ

By

Erling Sögnen

(Received August 3 1964)

It has been shown that lowering the plasma level of drugs by giving them orally along with calcium-binding substances (Ca-b.s.) by mouth is not due to increased renal excretion (SÖGNEN 1965 b)

To study the effects of Ca-b.s. on absorption, it is usual in the first place to use isolated intestinal loops *in situ* This method was first used by VERZAR (1936) and has since been employed by many workers with different modifications.

Material and Methods

Male rats weighing about 200 g were anaesthetized with urethane (1200 mg/kg i.p. of a 12 / (w/v) urethane solution) and placed on a thermostatically controlled table at 38° The abdomen was opened by a longitudinal incision along the midline. The intestinal segment where absorption was to be studied was isolated by ligation. The solutions were injected through the wall of the isolated segment by means of a thin (tuberculin) cannula. The site of injection was held between thumb and index finger for a short time after the injection. No leakage was observed. The abdomen was closed by continuous sutures in muscles and skin. After the end of the absorption period blood samples were withdrawn by cardiac puncture. Immediately afterwards the isolated intestinal segment was detached quickly from the mesentery cut out and weighed directly

In some experiments the contents of the intestinal loop were washed out with about 10 ml of distilled water and the loop was weighed before and after washing to determine the weight of the intestinal contents. The amount of sulphanilamide in the intestinal contents was determined after further dilution to 100 ml and protein precipitation in a 5 ml portion of this with the same amount of 7.5 trichloracetic acid. By this considerable dilution before protein precipitation, clear filtrates seemed to be more readily obtainable. The sulphanilamide determinations were performed as in the previous studies (SÖGNEN 1965 a).

To determine the initial amount of intestinal fluid (expressed as mg/cm intestine) animals in the experimental and the control groups were

killed immediately after the injection into the intestinal loop. The difference between the amounts initially and at the end of the absorption period indicates the amount of absorption or secretion that has taken place.

The initial amount of sulphanilamide was taken to be the same as that administered.

In these experiments the intestinal segments were not emptied of their original contents which were very small in fasting rats.

By this proceeding one is possibly working under more physiological conditions than when washing the segments with a 0.9 % sodium chloride or similar solutions before the experiments

Results

In table 1 are recorded the results of injections into small intestine loops ligated posterior to the stomach and anterior to the caecum. The solutions employed had the same concentration of Ca-b.s. as in the experiments on intact animals previously reported (SÖGNEN 1961 & 1965 a & b)

Table 1

Effects of equimolar amounts of tetracemin, sodium oxalate, sodium fluoride and sodium sulphate on the weight of isolated small intestine *in situ* of fasted rats.

1 ml/100 g rat of 0.1 M solution of Ca-b.s. in 0.9 % sodium chloride	Mean weight of small intestine from 2 rats after 2 hours, g	Weight of small intestine in % of animals weight
Tetracemin	12.2	4.8
Sodium oxalate	12.4	4.7
Sodium fluoride	14.8	5.9
Sodium sulphate 0.1 M	8.7	3.3
Sodium chlorid 0.9 %	8.1	2.8
Mean weight of 4 small intestines from intact rats	8.2	2.8

Table 2 illustrates the results of similar experiments with tetracemin The blood sulphanilamide concentration was here determined after the end of the absorption period.

Table 2

Blood concentration of sulphanilamide 1 hour after injection into the isolated small intestine of 1 ml of 1 % solution of sulphanilamide/100 g rat.

100 mg/kg sulphanilamide			100 mg/kg sulphanilamide 400 mg/kg tetracemin		
Blood concentration of sulphanilamide, µg/ml	Weight of small intestine in g	Weight of small intestine in % of animal weight	Blood concentration of sulphanilamide, µg/ml	Weight of small intestine in g	Weight of small intestine in % of animal weight
64	5.4	3.0	82	10.7	5.25
55	7.8	3.9	64	8.7	5.95
73	6.5	3.94	77	10.6	5.90
61	5.3	3.32	91	11.1	6.32

Tetraceminum (NFN) Edetic acid (BAN) Edathamil (NNR)

It is seen that the pronounced change in water transport did not result in a significant alteration of the sulphanilamide absorption, but there was a tendency towards increased sulphanilamide absorption in the presence of Ca b.s.

The increase in weight brought about by Ca b.s. is also demonstrable in isolated colon.

Table 3

Effect of tetracemin on colon weight and sulphanilamide absorption from isolated colon *in vi*

100 mg/kg sulphanilamide 1% sol.			+ 400 mg/kg tetracemin		
Weight of animal g	Weight of colon g	µg sulphanilamide/ ml blood	Weight of animal g	Weight of colon g	µg sulphanilamide/ ml blood
210	2.8	85	270	4.3	67
195	2.4	78	245	4.7	72
220	3.5	85	250	3.6	77
260	2.6	110	240	4.8	59
230	2.7	95	250	4.2	63
260	3.2	76	270	5.0	46
Mean	2.87	Mean 88.	Mean	4.43	Mean 63.5

Diff Sulphanilamide conc. = 4.7 µg/ml. $P < 0.01$
Period of absorption 2½ hours.

In these experiments a significant fall was seen in blood sulphanilamide level.

In theory the blood concentration of a substance is no reliable expression of the absorption from an intestinal loop On the other hand, these experiments were conducted with a view to finding the cause of the lowered blood level found in intact animals.

In the experiments whose results appear in table 4 sulphanilamide determinations were carried out both in blood and in the intestinal loops after the end of the absorption period. A more exact expression of the changes in fluid transport was also obtained by weighing the contents of the small intestine

It is seen in the table that considerable absorption of fluid took place from the isolated intestinal loop in the presence of calcium tetracemin, whereas in the presence of tetracemin the fluid content of the intestinal loop increased within one hour Sulphanilamide transport from the intestinal loop was reduced, but this did not show in the blood concentration of sulphanilamide.

Table 4

Absorption of fluid and sulphanilamide from loops of small intestine isolated *in situ* from the distal part of the ileum. Length of loop 15 cm. Amount of fluid injected 2 ml. Period of absorption 1 hour

		Intestinal content of		Blood concentration of sulphanilamide, µg/ml	Number of experiments
		Fluid mg/cm	Sulphanilamide, µg		
Tetracemin	Initial	156	2000		
10 mM	Final	181	318	21.07	13
	Absorbed	− 25	1682		
Calcium	Initial	150	2000		
tetracemin	Final	135	196	21.50	13
10 mM	Absorbed	15	1804		

Diff Sulphanilamide absorbed = 122 µg ($p < 0.001$).

Discussion

With sodium fluoride, sodium oxalate or tetracemin the volume of the intestinal loop was augmented either when the length of the absorbing area was kept constant, as in the experiments recorded in tables 3 and 4 or when the absorbing area varied with the supply of fluid to the intestine, as in the experiments in tables 1 and 2.

The experimental results recorded in table 2 are of particular interest in relation to choice of procedure in the study of the intestinal absorption from isolated intestinal loops By injecting 1 ml of solution/100 g, an initial absorbing area of about 15 cm in length was established, and the whole intestinal loop was 60–80 cm long. Owing to the anaesthesia, the tone and peristalsis of the intestinal loop were greatly reduced. The conditions were suitable for passive flow within the intestinal lumen if a larger fluid volume was supplied to the intestinal loop e g. by secretion into the loop

Such a supply of fluid took place in the experimental group whereas in the control group the volume was reduced owing to absorption

In the experimental group a large absorbing area was established and was exposed to a highly diluted solution compared with the more concentrated solution absorbed from a smaller area in the control group As the intraluminal pressure was also much greater in the intestinal loops of the experimental group than in those of the control group, many factors were here affected simultaneously

The experimental results were therefore of limited value in relation to the question of possible changes in membrane permeability

When the length of the absorbing area was not kept constant, there was a tendency towards a higher plasma concentration of sulphanilamide in presence than in the absence of tetracemin. This may have been due to increase in the absorbing area caused by Ca b.s.

In intestinal loops whose whole absorbing area is covered by the initial fluid amount, as in the experiments distally on the ileum (table 4) and on the colon (table 3), the absorbing areas also differ in the groups owing to distension of the loop by secretion. However, when the small intestine is involved, an approximately equal number of absorbing units (villi) will take part in the absorption in both groups.

Possible actions on the movements of the villi may also contribute towards masking other effects of Ca-b.s. on the small intestine. Such actions can be left out of account in experiments on the colon, which has no villi.

It is seen from table 3 that the blood concentration of sulphanilamide was lower after absorption from villus-free intestinal loops in the presence than in the absence of Ca-b.s.

Table 4 shows that in experiments with small intestine loops of a given length the absorption of sulphanilamide was reduced, without thus showing itself by a lowered blood sulphanilamide concentration. The fact may be due to an increased uptake of sulphanilamide by the intestinal tissue, but may also be accounted for by other alterations of sulphanilamide distribution, which cannot be discussed here.

SCHANKER & JOHNSON (1961) showed that the absorption of the fat insoluble drugs mannitol, inulin, a quaternary ammonium compound and sulphanilic acid were all markedly increased in the presence of tetracemin. They are normally not absorbed, and these authors suggest that tetracemin may act by increasing the size of the membrane "pores" or by widening the spaces between the epithelial cells through the removal of calcium ions.

The undissociated fraction of sulphanilamide is fat-soluble and will thus pass biological membranes through the cytoplasm of the cells. Widening of "pores" and intercellular spaces should therefore leave the transport of this compound unaffected or only slightly influences.

In our experiments, however, the Ca-b.s. caused a disintegration of the intestinal mucosa (ERICHSEN & SÖGNEN, unpublished results). This may have been so also in the experiments of SCHANKER & JOHNSON. It therefore seems justifiable to suggest that the absorption of macromolecular water-soluble substances is enhanced (or even made possible) when the mucosal epithelium is disintegrated, whereas the absorption of fat-soluble compounds, e. g. sulphanilamide, is slightly decreased or unaffected in such circumstances.

Summary

In experiments on isolated rat small intestine in situ it has been shown that the intraluminal application of calcium-binding substances, sodium oxalate, sodium fluoride and tetracemin cause an increase in the volume of intestinal fluid. The effect of calcium-binding substances on sulphanilamide disappearance from the loops depends on experimental conditions, but at any rate the effects observed cannot be responsible for the lowered plasma concentration seen in the intact animal.

Factors influencing absorption from isolated loops are discussed.

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Effects of Calcium-Binding Substances on Gastric Emptying as well as Intestinal Transit and Absorption in Intact Rats

By

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The inhibiting action of calcium-binding substances (Ca-b s.) on intestinal absorption is clearly demonstrable in the intact animal (SØGNEN 1961 & 1965 a).

On the other hand, experiments with isolated intestinal loops in situ have revealed that Ca b.s. cause scarcely detectable alterations in absorption from the small intestine (SØGNEN 1965 c) It is therefore reasonable to suppose that the actions of Ca-b.s. are in part associated with the functions of the stomach.

HUNT & SPURREL (1951) call attention to the relationship between gastric secretion and gastric motility and emptying.

When fluid is supplied to the stomach, the volume of fluid afterwards removed from this will exceed the volume added, owing to secretion (JAMES 1957).

As shown by HUNT & SPURREL (1951) and REYNELL & SPRAY (1956) gastric emptying is normally an exponential function of time.

Preliminary experiments showed that the volume of the gastric contents will increase in the presence of Ca-b.s. It was accordingly thought of interest to investigate the effect of this increase in volume on the emptying of substances from the stomach

As demonstrated by REYNELL & SPRAY (1956), the time of gastric emptying is related to the absorption rates of glucose and iodine.

The absorbing area and the time a substance is exposed to an absorbing surface affect the absorption This implies that a relationship is likely to exist between the rate of passage through the small intestine and intestinal absorption.

Material and Methods

Using REYNELL & SPRAY's method (1956) it is possible to study simultaneously both gastric emptying and intestinal transit and absorption. This method was therefore employed in these experiments.

As experimental animals fasting male rats weighing from 150 to 160 g were used. Though the procedure described was used in the main various modifications require further description.

Using phenol red, which is almost unabsorbed, as a marker we administered a test solution to rats. The distribution of the marker along the gastro-intestinal tract was used to calculate extent of gastric emptying, intestinal transit and absorption of the test substances.

Exploratory investigations showed that it was unneccessary to anaesthetize the animals before intubation. Intubation can probably be performed far more quickly with a glass tube, as is used in this Department, than with a rubber tube, as used by REYNELL & SPRAY

REYNELL & SPRAY killed their animals by a blow on the head. Desiring a more reflexless death, we preferred to anaesthetize the animals with ether before killing them. The rats were killed after opening the abdominal cavity for evisceration. The results of exploratory experiments showed phenol red to be distributed in the same way in the intestinal tract whichever method of killing was employed. Killing under ether anaesthesia, however has the advantage that blood samples can be withdrawn immediately before the end of the experiment without the animals being fixed or being exposed to pain which might result in peristaltic action (VERZAR & McDOUGALL 1926).

Solutions

Each animal was given approximately 1 ml/100 g body weight of a 0.9% sodium chloride solution containing 500 mg glucose/100 ml and 0.75 mg phenol red/ml. To meet this requirement and work at the same time with a constant amount of phenol red, rats of approximately equal weights (150 g) were chosen. Sodium oxalate, sodium fluoride, sodium phytate, *tetracemin and calcium tetracemin were administered as indicated below. The control and test solutions were made osmotically equal by means of sodium chloride.

Sulphanilamide was in most of the experiments added to the solution at a concentration of 1%, the dose of this drug being thus approximately 100 mg/kg. In calculating the plasma sulphanilamide concentration account was taken of the amount given per kilogram body weight. All the solutions had a temperature of 37° when administered.

The rats were killed as described above at the intervals after administration indicated.

The segments of the gastro-intestinal tract were ligated in the order pylorus, cardia and ileocaecal junction. The small intestine was taken out, by eviscerating first about 20 cm distally then the same length proximally and finally the remainder. During evisceration care was taken to keep the intestinal segments at approximately the same level, to prevent as far as possible displacement of the small intestine contents owing to differences in level. This was particularly important when intestinal segments containing fluid in the experimental group were taken out.

After evisceration the entire length of small intestine was transferred to a plastic channel graduated in centimetres. The total length was measured, and the intestine

* Tetraceminum (NFN) Edetic acid (BAN) Edathamil (NNR)

was divided in the middle. The stomach, ligated at the pylorus and the cardia, was taken out and, like the first and second segments of small intestine, transferred to small beakers, in which the segments were weighed immediately after evisceration.

If any phenol red had passed into caecum this was obvious on inspection. The detection was facilitated by injecting $\frac{1}{10}$ N ammonium hydroxide into the caecum, the stain being then more plainly visible through the intestinal wall. The stain is more difficult to see in the opened caecum. When no phenol red had passed into the caecum, the distance to this was determined by inspection and given in centimetres.

Homogenization and analytical method

As the analytical method indicated by REYNELL & SPRAY continued to give turbid filtrates, other protein precipitants were attempted. Trichloroacetic acid and absolute alcohol gave clear filtrates, but when these were rendered alkaline precipitates were seen in several of them. We therefore preferred to determine the phenol red concentration in an acid environment. After homogenization in 96% alcohol, the homogenate was acidified with concentrated hydrochloric acid, shaken and filtered. After suitable dilution, the phenol red concentration was determined spectrophotometrically at 510 mμ. The calculations were performed in relation to control values obtained on the basis of filtrates from homogenized intestinal segments of animals given solutions of tetracemin and calcium tetracemin free of phenol red one hour before killing. The calculations were made as indicated by REYNELL & SPRAY (1956).

Gastric emptying is defined as *the percentage of the administered phenol red that has left the stomach during any given period of time* and is calculated from the formula

$$\frac{Px - Pa}{Px}\ 100, \text{ where}$$

Pa = phenol red recovered from the stomach.

Px = phenol red recovered from the whole gastro-intestinal tract.

When phenol red was seen in caecum, this amount was calculated as the difference between the amount recovered from the stomach and small intestine in the particular animal and that recovered from the whole gastro-intestinal tract of controls. As control animals we used rats in which the passage of phenol red into the caecum was not demonstrated. The animals of the control group had all been killed 30 to 60 minutes after intubation. Our method differs also in this respect from that of REYNELL & SPRAY. They used control animals chosen in advance as a basis for calculation of the caecum contents. As their animals were killed immediately after the administration, and phenol red is known to be absorbed in small amounts, the choice of our controls is unlikely to be less appropriate than that of REYNELL & SPRAY.

Intestinal transit can be determined for each segment of small intestine separately for any adjacent pair of segments or for the small intestine as a whole. According to REYNELL & SPRAY (1956) the *transit through any segment is that percentage of the amount of phenol red entering the segment during the time since intubation which has moved on to the next segment during the same period of time*. Thus, the transit through the first half of the small intestine is given by the formula

$$\frac{Pd}{Pb + Pd}\ 100, \text{ where}$$

Pb = phenol red recovered from the first small intestine segment.

Pd = phenol red recovered from the intestinal portion distal to the first segment, including the amount passed into the caecum.

As the absorption itself in the presence of Ca-b.s. has been investigated by various other methods (SÖGNEN 1961 & 1965 a, c & e), this was not examined other than by measuring the blood concentration of sulphanilamide after the end of absorption period.

The method employed for sulphanilamide determination has been given in a previous paper (SÖGNEN 1965 a).

Results

Distribution of phenol red in the gastro-intestinal tract

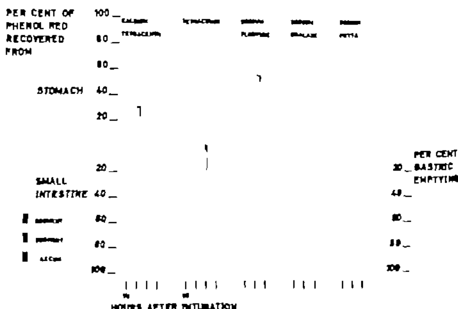


Fig. 1 The columns demonstrat gastric emptying and distributio of phenol red in the gastro-intestinal tract t different times after intubatio of 1 125 mg phenol red. Numerical values given in table 1

Table 1 and fig. 1 illustrate the distribution of phenol red in the individual segments of the gastro-intestinal tract.

All the Ca b.s. were at any time after intubation seen to have caused a reduction in phenol red content of the small intestine, compared with that of the control group (calcium tetracemin) The part of the intestinal tract from which most substances have the best chances of being absorbed, and the one with the largest absorbing surface, will, in other words, be at any time exposed to smaller amounts of substance in the experimental than in the control group

Table 1

Percentage of total dose of phenol red recovered from different segments of the gastro-intestinal tract of rat t varying times after intubation. Total dose of phenol red 1 125 mg in 1.5 ml 0.1 M solutions of calcium tetracemin (control), tetracemin (372 mg/kg) sodium floride (42 mg/kg), sodium oxalate (134 mg/kg) and sod um phytate (1000 mg/kg).

		Hours after int bation							
		½		1		2		3	
			mean		mean	n	mean	n	mean
Calcium tetracemin (Control)	Stomach	5	48.4 ± 4.4	6	30 0 ± 5 1	6	12.2 ± 3.2	5	13 5 ± 3.6
	Segment I	5	30.5 ± 3 4	6	18.6 ± 3.2	6	11.8 ± 4.0	5	7.0 ± 1 4
	Segment II	5	21.0 ± 1 7	6	51 5 ± 6.5	6	58 8 ± 3.8	5	55.0 ± 5.9
	Caecum	5	0 0	6	0 0	6	18.9 ± 4 7	5	4 9 ± 5 6
Tetracemin	Stomach	6	71.5 ± 3 6	6	71 1 ± 4.5	5	58.7 ± 6.2	6	38.8 ± 6.2
	Segment I	6	12.1 ± 1.8	6	8.0 ± 1.5	5	9.9 ± 3.0	6	6.5 ± 7.9
	Segment II	6	16.4 ± 3 6	6	13 3 ± 1 1	5	8.4 ± 1 0	6	1..8 ± 3 1
	Caecum	6	0 0	6	7.6 ± 4 0	5	20.2 ± 4.3	6	4..0 ± 4.4
Sodium fluoride	Stomach			3	67.8 ± 3.4	3	54 6 ± 2.8	3	32.6 ± 9.5
	Segment I			3	14 8 ± 4 1	3	19 1 ± 4.8	3	13 1 ± 3 9
	Segment II			3	17 4 ± 0.9	3	20.2 ± 4.2	3	24.6 ± 8.2
	Caecum			3	0 0	3	7.2 ± 3 6	3	29 6 ± 5.3
Sodium oxalate	Stomach			3	57 1 ± 6.6	3	38.2 ± ..9	3	34.7 ± 7.5
	Segment I			3	12.6 ± 0.8	3	9 6 ± 2.6	3	11.2 ± 2.7
	Segment II			3	30.3 ± 5.8	3	34.2 ± 9 6	3	33.7 ± 5.2
	Caecum			3	0 0	3	9 ± 9.3	3	20.3 ± 8.3
Sodium phytat	Stomach			3	48.3 ± 4.1	3	24 4 ± 7 1	3	15.2 ± 5.4
	Segment I			3	15 1 ± 2.2	3	8.9 ± 0.3	3	9 4 ± 1 3
	Segment II			3	36.4 ± 2.2	3	28 0 ± 10.0	3	23 6 ± 1 7
	Caecum			3	0 0	3	31 7 ± 15 9	3	51.8 ± 4.7

After two or three hours, the reduced content of phenol red *in the* small intestine was due in part to a relatively larger content of phenol red in the caecum of the experimental animals than in the controls.

Determining *the phenol red distributions at different times after intubation*, it was seen that after 30 minutes more phenol red was recovered in the stomachs of the experimental animals (the tetracemin group) than in those of the controls (the calcium tetracemin group) Further it was noticed that the control group had the largest amount of phenol red in the first half of the small intestine (Seg. I), whereas the second half (Seg. II) contained the largest amount in the experimental group No phenol red had by this time passed into the caecum in any of the groups.

After 1 hour all the experimental groups could be compared with the control group. First, the fact was established that in the tetracemin group the phenol red content of the stomach remained practically un-

changed during the 30 to 60 minutes after intubation. Nevertheless, of the small amount of phenol red that had moved on to the small intestine, a minor proportion had passed into the caecum in all six animals of the group In the control group the phenol red content of the stomach had fallen about 20 / within the 30 to 60 minutes after intubation, constituting two-thirds to half of the phenol red contents in all the experimental groups

After 2 and 3 hours there still was more phenol red in the stomachs of all the experimental groups than in those of the control group.

After 1 2, and 3 hours much more phenol red was recovered from Seg. II than from Seg. I of the small intestine in the control group The situation was similar in the experimental group but the difference in the segmental distribution of phenol red was smaller there than in the control group

Gastric emptying

Fig. 1 and 2 illustrate the effects of Ca b.s. on gastric emptying.

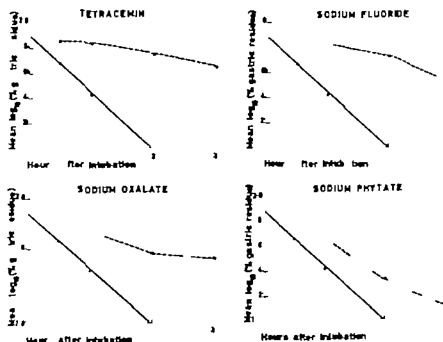


Fig. 2. Gastric emptyi g affected by equimolar amounts of calcium-binding substances. Experimental ●——● Control ○——○
The diagram indicates the mean logarithm of the percentage of the initial phenol red dose remaining the stomach, plotted against time after intubation.

It is seen that tetracemin and sodium fluoride have approximately the same depressing effect on gastric emptying when given in equimolar amounts. Sodium oxalate holds a position intermediate between these and sodium phytate, which has the least depressing effect on this function.

Rate of passage through the small intestine

The transit can be calculated on the basis of the distribution of phenol red in the various gastro-intestinal portions. However such a calculation affords no exact indication of how far the phenol red column has passed through the small intestine. Information on this is best obtained by direct inspection of the small intestine before the phenol red column has reached the caecum and after the intestine has been taken out for division into segments.

Table 2

Distance of phenol red column from caecum 30 and 60 min. after intubation.

Calcium binding substance	Distance from caecum (cm)					
	30 minutes			60 minutes		
	mean		s.e.m.	mean		s.e.m.
Calcium tetracemin (Control)	29.2	±	3	11.2	±	3.75
Tetracemin	15.3	±	4.41	Phenol red in caecum		
Sodium fluoride				0		0
Sodium oxalate	–		–	4.67	±	0.97
Sodium phytate	–		–	6.67	±	1.67

The table shows that all Ca-b.s. accelerate the passage through the small intestine. When, as in our experiments, they are given in equimolar amounts, the rate of passage, like gastric emptying, is affected to the greatest extent by tetracemin and sodium fluoride and to the least by sodium phytate and sodium oxalate.

Transit

After half an hour a tendency towards increased transit in Seg. I was noticed in the tetracemin group (table 3).

After 1 hour the transit in Seg. I was unaffected in all the experimental groups, except the sodium fluoride group, in which a tendency

Table 3

Intestinal transit in absence and presence of calcium binding substances calculated for the first and the second halves of the small intestine and for the whole small intestine.

Calcium binding substance	Transit through small intestine	Hours after intubation ½ n	½ mean	1 n	1 mean	2 n	2 mean	3 n	3 mean
Calcium tetracemin (Control)	Segment I	5	41.3 ± 2.6	6	72.3 ± 5.6	6	86.6 ± 4.3	5	91.3 ± 3.5
	Segment II	5	0 0	6	0 0	6	33.3 ± 5.2	5	30.2 ± 6.5
	Whole	5	0 0	6	0 0	6	20.7 ± 5.0	5	28.0 ± 6.3
Tetracemin	Segment I	6	54.4 ± 8	5	70.1 ± 3.3	6	74.3 ± 5.9	6	89 ± 6.2
	Segment II	6	0 0	5	49.8 ± 9	6	68.4 ± 4.3	6	75.2 ± 6.9
	Whole	6	0 0	5	34.9 ± 7.3	6	51.7 ± 5.9	6	67.7 ± 7.4
Sodium fluoride	Segment I			3	55.6 ± 7.7	3	60.7 ± 3.8	3	79.5 ± 7.3
	Segment II			3	0 0	3	26.9 ± 13.6	3	56.7 ± 8.4
	Whole			3	0 0	3	16 ± 9	3	45 ± 7.9
Sodium oxalate	Segment I			3	69.9 ± 2.4	3	84.4 ± 0.5	3	81.6 ± 5.6
	Segment II			3	0 0	3	34.7 ± 17.5	3	35.7 ± 11.3
	Whole			3	0 0	3	29.4 ± 7.2	3	29.7 ± 10.3
Sodium phytate	Segment I			3	71.9 ± 3.6	3	88.1 ± 0.8	3	88.8 ± 2.8
	Segment II			3	0 0	3	54.3 ± 6.7	3	68.6 ± 1.5
	Whole			3	0 0	3	48.1 ± 18.7	3	60.9 ± 2.7

towards a reduced transit was noticed. After this interval, transit through Seg. II was only observed in the tetracemin group

After 2 hours the transit through Seg. I was practically unaffected, except in the sodium fluoride group, in which it was somewhat reduced. Through Seg. II there was a clear tendency towards increased transit in the experimental groups. The increase was the least pronounced in the sodium fluoride group (with individual observations of 37 0 and 43) and in the oxalate group (with observations of 55.9 48.3 and 0). In other exploratory experiments, there had always been transit after 2 hours in the experimental groups The observation of no transit in one of three experimental animals therefore cannot be said to be characteristic at this time after administration of sodium oxalate and sodium fluoride. For the same reason the tendency towards increased transit through the entire small intestine was smaller than usual in the sodium fluoride group (with observations of 25 0, and 23) and in the oxalate group (with observations of 47 41 and 0)

After 3 hours the transit through Seg. I was still unaffected. Through Seg. II and through the whole small intestine it was increased in all the

experimental groups. In the sodium oxalate group the variation was great and the increase not significant.

Considered collectively Ca b.s. caused increased transit through the posterior segment of the small intestine and through the small intestine as a whole.

Weight increases of the stomach and intestinal segments

The gastro-intestinal segments were weighed after evisceration. The results are recorded in table 4.

It is seen that in all the experimental groups the stomach as well as Seg. I and Seg. II of the small intestine showed a considerable increase in weight at most intervals after the administration. The differences between the segment weights in the experimental groups and the control group were greater than the weight of the volume of solution given (1.5 ml/rat). This means that an increased secretion took place in the experimental animals, which was not counterbalanced by absorption from the intestine.

The considerably increased stomach weights showed that the volume of gastric secretion exceeded that passing to the small intestine during the same period.

Whether the increases in weight of the intestinal segments were due solely to transport of secretion from the stomach, or whether the small intestine secretion also was increased could not be established by this experiment. The effects of Ca b.s. on water transport in isolated small intestine loops *in situ* are demonstrated in a previous paper (SÖGNEN 1965 c). The transit of ingesta to the caecum and colon within the observation period however caused the difference in weight between the gastro-intestinal tracts of the experimental group and of the control group to be much smaller than it would have been if the caecum and the colon had been included in the calculations. In contrast to the stomach and small intestine, these intestinal portions are not emptied after 24 hours fasting. Their weights therefore vary appreciably and much material would be required to demonstrate by weighing possible effects on the volumes of these portions.

Table 4 shows that during the first two hours after administration the weights of the gastro-intestinal segments in the experimental group increased in comparison with those of the control group. The difference in weight was found to decrease with time, being eliminated in the tetracemin group after 3 hours for Seg. I and Seg. II. The same was true of Seg. I in the sodium fluoride group and the sodium phytate group after the same interval.

Table 4

Mean weights of stomach and small intestine and mean weight

Hours after

Calcium-binding substance	½				1			
	Stomach	Small intestine		Stomach + small intestine	Stomach	Small intestine		Stomach + small intestine
		Seg. I	*Seg. II*			*Seg. I*	*Seg. II*	
Calcium tetracemin	2.37	3 68	2.95	9 0 ± 0.58	2.05	3 6	3.87	9.52 ± 0.11
Tetracemin	3.27	3.87	3.98	11 12 ± 0 49	3.50	4 05	4.55	12.10 ± 0.30
Difference	1.90	0 19	1 03	2.12	1.45	0.45	0.68	2.58
Sodium fluoride					4 67	4.91	4.93	14.51 ± 0.41
Difference					2.62	1.31	1.06	5.59
Sodium oxalate					3 16	3 17	3 47	9.8 ± 0.18
Difference					1 11	0.57	0.60	0.[illegible]
Sodium phytate					3.39	3.55	4.33	11.27 ± 0.34
Difference					1.34		0.46	2.35

Sulphanilamide concentration in plasma

The effect of Ca b.s. on plasma concentrations of sulphanilamide has been dealt with previously (SÖGNEN 1965 a) The table shows the effect

Table 5

Sulphanilamide concentrations in plasma after different time intervals. Dose of sulphanilamide approxim tely 100 mg/kg.

Hours after intubation

Calcium binding substance	½		1		2		3	
	mean s.e.m.	% acetylation	mean s.e.m.	% acetylation	mean s.e.m.	% acetylation	mea s.e.m.	% acetylation
Calcium tetracemin (Control)	68.9 ± 11 7	12	74 3 ± 3.5	14.9	69.3 ± 2.2	19.5	58.9 ± 1.5	29.9
Tetracemin	41 6 ± 3	19 4	37 7 ± 2.6	32.8	40.7 ± 2	33.9	48.7 ± 3 9	34.8
Sodium fluoride			42 ± 6.3	36.3	32.3 ± 6.7	48.1	52.1 ± 2.6	38
Sodium oxal te			55.3 ± 9 6	20.7	55.4 ± 5.9	23.3	54.2 ± 1.6	36.7
Sodium phytate			44 4 ± 4.2	23 5	57 7 ± 4.3	2[illegible]	± 0.8	33

differences between control and experimental groups, expressed in g.

intubation

2				3			
Stomach	Small intestine		Stomach + small intestine	Stomach	Small intestine		Stomach + small intestine
	Seg. I	Seg. II			Seg. I	Seg. II	
2.28	3.16	3.15	8.58 ± 0.52	[illegible].09	3.58	3.53	9.2 ± 0.61
3.67	4.03	3.53	11.23 ± 0.64	3.45	3.56	4.41	11.42 ± 0.36
1.39	0.87	0.38	2.65	1.36	−0.02	−0.[illegible]	2.22
3.72	3.97	4.54	12.23 ± 0.29	3.26	3.55	4.1	10.91 ± 0.77
1.44	0.81	1.39	3.65	1.17	−0.03	0.57	1.71
3.13	3.68	3.71	10.52 ± 0.3	2.72	4.19	4.05	10.96 ± 1.17
0.85	0.52	0.56	1.94	0.63	0.61	0.52	1.76
3.04	3.43	4.31	10.78 ± 0.68	2.94	3.54	3.76	10.[illegible]4 ± 0.32
0.76	0.32	1.16	2.30	0.85	−0.04	0.23	1.04

to be the same in principle in the presence of phenol red. It is seen how the acetylation percentage was secondarily affected by Ca-b.s., the low plasma levels being associated with a high acetylation percentage.

As to reducing the plasma sulphanilamide concentration, tetracemin and sodium fluoride also have a greater effect than sodium oxalate or sodium phytate

Discussion

As will be seen in table 4 Ca-b.s. markedly increase the stomach weights. This is due to a considerable increase in gastric secretion, which begins rapidly after oral administration of Ca-b.s. This secretion is the immediate cause of the retarded emptying of phenol red from the stomach, because the volume of gastric fluid increases more rapidly as a result of secretion than the gastric contents can be removed from the stomach to the small intestine. In other words, the dilution of the initial solution of phenol red implies that less phenol red leaves the stomach per unit of time

Preliminary experiments (SÖGNEN, unpublished) seem to reveal that gastric pH will rise in presence of sodium fluoride and will fall in presence of sodium oxalate since both substances cause the same changes in rate of secretion and gastric emptying, it is unlikely that pH alterations have anything to do with the effects being considered here

The nature of this secretion will, however not now be further examined. In the further examination of the problem, however it may be of some value briefly to review some facts related to gastric emptying and secretion that may be of importance in the present investigation. They are 1 the pattern of stomach emptying 2. the relation between calcium and gastric secretion 3 the relation between gastric secretion and gastric emptying 4 intestinal effect on gastric emptying, and 5 the effect of calcium on intestinal motility

1 *The pattern of stomach emptying*

The works of several authors reviewed by JAMES (1957) show that, at least for a liquid meal, the volume emptied during any interval of time after the meal exceeds the volume secreted and that the combined volume is therefore at a maximum immediately on completion of a meal and then lessens progressively

In the presence of Ca b.s., however this is not so As will be seen from table 4 the volume of the stomach increases. The effect of this increase in secretion has been further examined by means of the method first introduced by HUNT & SPURREL (1951) They plotted the volume of the meal remaining against time Plotting the logarithm of the amount remaining against time gave a straight line with a downward slope, indicating that the disapperance of a meal from the stomach is exponential. An exponential disappearance can be described by the "half life that is, by the time taken for the amount remaining to be halved, which will be the same throughout the periode of disappearance.

When Hunt and Spurrel extrapolated back to the log of the initial value of the volume of the meal, the line did not always reach that value at zero time (the time of administration) The initial value might be reached earlier or later than at zero time. This means that sometimes the exponential pattern of stomach emptying is not assumed immediately after administration of the meal indicating that sometimes the stomach will empty at a more rapid initial rate or at a slower rate. These deviations are called by the authors negative and positive starting index, respectively

In our experiments it was not a question of the volume of the meal remaining, but of the amount of phenol red remaining in the stomach at different times after intubation. As will be seen from fig. 2, extrapolation of the semilogarithimic line in the control group indicates that the log of percentage gastric residue is less than 2 at zero ti [illegible] indicates a negative starting index in rats after [illegible] This seems reasonable since some of the [illegible] the duodenum in connection with the [illegible]

In the experimental groups the [illegible]

stomach is markedly retarded. For sodium phytate, however there is an approximately normal pattern with a positive starting index.

Since the residue of phenol red is not reduced in the interval between 2 and 3 hours, gastric emptying is considered to be finished after 2 hours.

These results do not give information about the volume of fluid leaving the stomach per unit of time. Since secretion is markedly increased (table 4), it may well be that more fluid is leaving the stomach per unit of time than under normal conditions. This might be examined by measuring concentrations of phenol red (HUNT & SPURREL 1951) or by a fistula in the duodenum, but to use the last would necessitate anesthetizing the animals, and anaesthesia influences stomach emptying (SÖGNEN, preliminary experiments). Another difficulty that might compromise the results, obtained by the latter method would be an alteration in intraluminal pressure in the intestine. In presence of a fistula the pressure in the duodenum would fall and thus the intestinal phase of the gastric secretion might be affected.

2. *The relation between calcium and gastric secretion*

The relation between alterations in blood calcium levels and rates of gastric secretion has been studied by GRANT (1941 a). The author cites the conclusion of BABKIN & KOMOROW (1940) in their review on this subject. "The data concerning the effect of hyper and hypocalcaemia on the nervous and humoral regulatory mechanism of the gastric glands are few and contradictory." GRANT has, however in several experiments, to be reviewed here, contributed to the solution of this and closely related problems.

In the first series of experiments on dogs, the author stated that after intravenous injections of calcium chloride and calcium lactate the rate of gastric secretion was reduced by an average of 74 %. The secretory response to both histamine and vagues stimulation was enhanced by intravenous injections of calcium. Alterations in blood pressure and gastric blood flow as well as changes in osmotic pressure, were excluded as responsible for the reduced secretory response.

According to the results of several authors reviewed by GRANT (1941 b), the calcium content of the gastric secretion varies considerably with the experimental conditions. In 11 normal dogs it was found to be 6.08 mg calcium/100 ml. In the fasting secretion of a pregnant dog the calcium ranged from 11.07 to 14.8 mg/100 ml and in histamine induced secretion it was 11.07. In fundic pouch secretion of 4 dogs the calcium content was much lower 1.10 to 3.30 mg/100 ml. In cats the juice from Heidenhein pouches was found to contain 4.1 mg in the fasting state, 10.6 mg 2–4 hours after food and 5.7 mg/100 ml 8 to 12 hours after food.

The most obvious result of GRANT's experiments was *the reciprocal* relationship between calcium concentration and the acidity of the gastric secretion in dogs.

Evidence that high acidity is accompanied by low calcium, and vice versa, was seen by comparing the levels of calcium and acidity in 24 animals and in single experiments, when vagal and histamine stimuli were used alternatively to produce their characteristic secretions of low or high acidity respectively Finally the fall in acid gastric secretion after intravenous injection of calcium was demonstrated.

Further experiments (GRANT 1942) led to the following conclusions Mucus from surface epithelial cells and from the cells producing it in the vagal type of secretion is an immediate source of calcium in gastric secretions. Calcium is liberated from the mucus in a reaction associated with the reduction in acidity

The general conclusion from Grant's experiments should, however be that high systemic calcium results in high concentrations of calcium in gastric mucus secretion and causes a simultaneous reduction in gastric acidity and rate of gastric secretion. The fall in acidity is, however not due to the reduced volume rate of secretion.

There may be a connection between Grant's experiments with high calcium and reduced secretion and our experiments with low gastro-intestinal calcium and increased secretion, but the effects of Ca-b.s. may equally well be due to a more unspecific stimulation of gastric secretion. HOLLANDER (1962) gives a review of the physiology and chemistry of the secretion of gastric mucus and mentions the stimuli that cause such secretion 1) electrical stimulation of splanchnicus or vagus 2) subcutaneous injection of cholinergic drugs, e. g. pilocarpine or mecholyl. High dosages give alkaline viscous mucus Low dosages give viscous nonacid mucus at first and later HCl and pepsin secretion combined with mucus

Topical applications of pilocarpine, mecholyl and acetylcholine give mucus of relatively high viscosity whereas intra-arterial injection of acetylcholine close to the gastric wall yields an acid mucoid fluid or a mixture of hydrochloric acid and mucus.

Topical stimulation by various irritant solutions, e. g. 3–5 / eugenol *(allyl-guajacol), gives an opaque mucus of exceptionally* high viscosity and high contents of columnar epithelium and leucocytes.

Mechanical stimulation (rubbing) gives a qualitatively similar response, but with a much lower volume rate of output and lower cell content.

Topical application of various other substances – iodoacetamid, N-ethylmalimid, ethylisothiocyanate, phenergan, nupercain among them – yields a mucus fluid of moderately high viscosity and entirely free of cells.

The latter reactions may be considered as a protection against chemical irritation that might be caused by the Ca-b.s. independent of their calcium-binding properties. There are, however, no such reactions in presence of the calcium salts of fluoride or tetracemin.

3 *Gastric secretion and gastric emptying*

Since in the work reported here an increase in secretion and a marked retardation in emptying of phenol red from the stomach were simultaneously observed, it seems justified to give a brief review of the relationship between secretion and gastric emptying.

It is now clear that the pylorus act as an integral part of the antrum and not as an independent sphincter controlling the exit of gastric contents (ATKINSON *et al* 1957, EDWARDS 1961, ARMITAGE & DEAN 1963) but the theory of acid control of the pylorus introduced by CANNON (1904) is still valid.

The fact that a strongly acid reaction can "close the pylorus" (or as it ought to be expressed now, cause contraction of the pyloric part of antrum) has been confirmed by MCILVAIN (1923), VAN VIERE & SLEETH (1940), SHAY & GERSHON-COHEN (1934) and PATHAK (1958).

The last-named studied the gastric response of human subjects to various concentrations of HCl by HUNT's method (1951) with phenol red as a marker. He found that HCl at concentrations up to 10 mEq/l seemed to favour gastric emptying, whereas with increasing concentrations above 10 mEq/l there was a progressively larger retention of gastric contents. As stated by GRAY & BUCHER (1941), the output of HCl from the gastric glands increases with the rate of secretion in a linear fashion, but not necessarily at the same rate. In our work the marked increase in gastric secretion might accordingly have been expected to cause a simultaneous increase in output of secreted HCl, but, as already mentioned, the pH values of gastric contents may increase in presence of one type of Ca b.s. though it decreases in presence of another. It is therefore unlikely that the observations made in our investigation are basically related to alterations in pH.

4 *Intestinal effect on gastric emptying and secretion*

To observe whether some intestinal mechanism may be involved in the results observed by us, some preliminary experiments were carried out in which the Ca-b.s. were introduced into the duodenum, which was isolated from the stomach by ligature. An increase in gastric secretion did not take place (SÖGNEN unpublished). It is therefore justifiable to omit discussion of intestinal factors affecting gastric secretion and motility such as the effect of Ca-b.s. on the osmoreseptoric zone, which is

presumed to be situated at or beyond pylorus (HUNT 1956 1960 and HUNT & PATHAK 1960) Similarly the influence on gastric secretion through increased pressure in the intestinal lumen (SIRCUS 1953) may be excluded from this discussion

5 The effect of calcium on intestinal motility

It has been postulated that calcium ions act as the link between depolarization and contraction in cardiac (NIEDERGERKE 1959) and skeletal (FRANK 1958) muscle Thus hypothesis is based on the results of experiments relating the effects of depolarization with various concentrations of calcium ions ROBERTSON (1960) has demonstrated in experiments with tetracemin that the presence of calcium in the bath fluid is necessary for the contraction produced by acetylcholine in isolated smooth muscle already depolarized with potassium sulphate. The experiments tend to confirm the importance of calcium ions at the membrane DURBIN & JENKINSON (1959) suggest the possibility that acetylcholine increases membrane permeability to calcium as well as to monovalent ions.

If the intraluminal presence of Ca b.s. deprives intestinal smooth muscle of calcium ions, it is therefore likely that this will contribute to decreased tone and peristaltic movement.

Systematic calcium, however must be assumed to be little influenced by intraluminal application of Ca-b.s. Since calcium ions must be supplied to the intestinal smooth muscle by circulation, this direct action on intestinal motility is rather unlikely to occur in vivo In vitro however it is clear that the absence of calcium ions decreases intestinal tone and peristaltic movements

Examination of the mechanism behind the increased rate of intestinal transit in our investigation will require further experiments including examination of the effect of Ca b s on intestinal peristalsis It is, however generally accepted that intraluminal pressure should stimulate to peristalsis. But whether such increased peristalsis can take place in presence of Ca b.s. is uncertain. The increased rate of intestinal transit may here be due to a passive flow of fluid whose rate is increased because of the increased intraluminal hydrostatic pressure

Conclusion

It seems evident that the observations provide no basis for further discussion of the mechanisms behind the alterations in rate of secretion, gastric emptying and intestinal transit.

The observations may however be of general importance in toxicology and for problems of retarded absorption, since other toxic substances

may produce similar reactions in the gastro-intestinal tract (SÖGNEN, unpublished) and cause a retardation of their own absorption.

The possibility also exists that the mechanism in certain circumstances favours detoxification processes by reducing the rate at which the toxic agent is introduced into the organism. This might be of special value when the detoxification process is of limited capacity With sulphanilamide it is seen that the proportion of acetylated sulphanilamide is larger during the initial period of absorption, when the plasma concentration of sulphanilamide is low (table 5)

Summary

It has been demonstrated that the reduced gastro-intestinal absorption of drugs in presence of calcium-binding substances is mainly due to a marked decrease in the rate of gastric emptying, but probably also partly to an increased rate of passage through the small intestine by the contents coming from the stomach.

The immediate cause for the retarded rate of gastric emptying is a considerable increase in gastric secretion.

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Effects of Calcium-Binding Substances on Glucose and Sulphanilamide Absorption from Perfused Small Intestine Loops In Situ

By

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It has been shown in an earlier paper (SØGNEN 1965 c) that calcium-binding substances (Ca-b.s.) cause fluid to be transported to the isolated intestinal loops *in situ*. This involved a reduction in the initial concentration of the substances whose absorption is to be studied. On the other hand, the transport of fluid to the intestinal loop results in an increased absorbing area and a rise in intraluminal pressure in the loop. These two latter actions may contribute towards masking any possible effect on transepithelial translocation of substances by Ca-b.s.

With SHIFF & SMYTH's method (1955) variations in the absorbing area and pressure conditions can be practically eliminated.

Material and Methods

Fasting male rats weighing from 200 to 250 g were used. These were anaesthetized with intraperitoneal urethane, 1200 mg/kg, 10 minutes before the surgical intervention. SHIFF & SMYTH used pentobarbital sodium (mebumal sodium) as anaesthetic. In the experience of this Department urethane is a suitable anaesthetic for both rats and guinea-pigs, whereas pentobarbital tends to give complications in rats: mucus production in the respiratory organs, among others.

The urethane-anaesthetized rat was placed on a table heated to 38°. The abdominal cavity was opened and the intestine cut at duodenojejunal flexure. The small intestine was washed with physiological saline at a temperature of 38°.

Procedure (see fig. 1)

The abdomen of the anaesthetized animal is opened. The part of the intestine to be perfused is isolated by ligatures. It is emptied and then washed through with physiological saline.

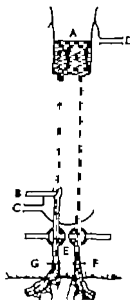


Fig. 1 Apparatus used for circulating fluid through intestine (SHEFF & SMYTH 1955). Fluid contained in reservoir A is circulated through intestine by means of gas-lift B. Warm water (38°) enters apparatus t C and leaves at D Two-way taps at E facilitate rapid washing of ntestine.

The oral end of intestine is connected at F with the apparatus described above and the distal at G The gas (in the original method O_2, in our experiments air) from B will then cause to circulate the fluid from the reservoir A through the animal's intestine.

After perfusion is concluded, the intestine and the reservoir are washed out quickly with 200 ml of physiological saline by means of the two-way tap E.

From the known concentration of the substance whose absorption is to be studied and the final concentration determined after 20 minutes, for instance, the amount of substance absorbed is calculated.

With the original method, the rat is placed on a heat-regulated table during the perfusion. In our experiments the rats were laid on a Roux bottle, which was pushed into the same circulating system as is used for heating the jackets surrounding the perfusion vessel and the air in the gas-lift. The air is supplied from an Air Master tank oxygenator and passes through the fluid in heat-regulated washing bottles before reaching the gas-lift.

After the small intestine contents have been pressed into the caecum, the small intestine was ligated and partially cut through immediately anterior to the ligature. It was then attached to the connecting piece and washed further with 10 ml of 0.9 / sodium chloride solution. The rest of the wash fluid was blow out of the intestine with a pipette, and the small intestine was connected with the perfusion vessel by means of the mounted connecting pieces.

The perfusion fluid was put into the perfusion vessel and heated to 38° The perfusion was then began after the rat's rectal temperature had been recorded. Urethane anaesthesia, like other forms of anaesthesia, causes hypothermia. However by placing the animal on a heated table as soon as possible after anaesthesia occurs

and washing with fluid at 38° the body temperature can be kept constant within ± 1 degree of normal.

In these experiments the perfusion fluid circulated by means of air from a tank oxygenator SHEFF & SMYTH used oxygen in the gas-lift. As in the original method, the air was saturated with moisture to avoid loss by evaporation of fluid from the perfusion vessel.

Perfusion took place under a pressure of 20 cm water According to FISHER & PARSONS (1949) this should give sufficient distension to expose the entire mucous membrane to the perfusion fluid. The circulation in the system can be controlled by following small particles in the ascending and descending brackets of the perfusion apparatus. The experienced worker will rarely encounter any hold-up in the circulation of the system. If he does, the circulation can usually be easily restored by squeezing the intestinal loop or by straightening out the obstructed part carefully

The perfusion periods were usually 20 min., interrupted by a resting period of 20 min.

After the end of perfusion, the fluid in the intestine and apparatus was washed out with 0.9 sodium chloride, from a reservoir kept at 38° to give a total final volume of 200 ml.

During washing of the intestine before and after the experiment, the hydrostatic pressure was kept under 60 cm. If the pressure is raised beyond this, the intestine may be damaged (WISEMAN 1961).

At the end of the experiment the small intestine was eviscerated and everted over a glass rod for inspection, with a view to detecting grossly visible morphological changes. Further representative specimens were taken for histological examination.

Solutions

Where not otherwise stated, 30 ml of 0.9 / sodium chloride solution containing 1 mg/ml glucose and 3 mg sulphanilamide were used for the perfusion. When a Ca-b.s. was used in the second perfusion period, this was contrabalanced in the first by adding equimolar amounts of sodium chloride.

Immediately after the end of perfusion, thrichloroacetic acid was added to a measured portion of the perfusion fluid for protein precipitation. The mixture was shaken, filtered and left standing for analysis.

The sulphanilamide determinations were performed as previously described (SÖRENSEN 1965 a). The glucose determinations were carried out by HALSE's modification (unpublished) of PROKHOVNIK & NELSON's method (1953). Instead of a burette, we used for measuring anthrone solution a syringe delivering in a fixed volume.

Results

A hypothetical effect of Ca-b.s. on the transport of sulphanilamide *might* bear a relation to a simultaneous effect on water transport caused by deficient utilization and transport of glucose (see discussion). Preliminary experiments were therefore conducted with *perfusions of a fluid* in which glucose had been replaced by xylose. According to LIFSON & PARSON (1957), water transport depends on the absorption and metabolism of glucose. It was therefore a matter of interest to study the transport

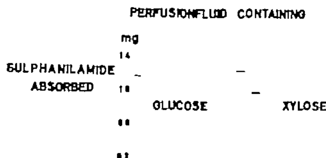


Fig. 2. Sulphanilamide absorption from solutions of equimolar concentration (27.75 mM) of glucose and xylose during a single absorption period of 30 min. Initial amount of sulphanilamide 3 mg in 30 ml of saline. The columns give mean values for 9 animals in each group. The difference is significant. $P < 0.001$

of sulphanilamide in the absence of glucose. The result is illustrated graphically in fig. 2.

In table 1 are recorded the effects of Ca-b.s. on the transport of glucose and sulphanilamide.

The transport of glucose is seen to be slightly reduced in the experimental group as compared with the control group. A clear depression in absorption was only noticeable in the presence of high concentrations of sodium fluoride or sodium oxalate. (Sodium oxalate caused no depression at a concentration of 10 mM.) The depression in the presence of sodium fluoride is in some measure related to the morphological changes this substance produces at the concentrations used. Tetracemin and sodium oxalate, on the other hand, caused no morphological changes (ERICHSEN & SÖGNEN unpublished). It is seen that Ca b.s. had no effect on the transport of sulphanilamide under these experimental conditions. Only sodium fluoride inhibited the transport to some extent, but morphological changes were found here. It has, in other words, been shown that a damaged mucosa is less permeable to sulphanilamide than an intact one. Perfusions for one period per rat showed exactly the same tendencies as those demonstrated in table 1.

The sodium fluoride and sodium oxalate used in the perfusion will cause precipitation of the respective calcium salts, which may affect the mucosa mechanically.

In exploratory experiments the transport of glucose and sulphanilamide was therefore studied in the presence or absence of calcium in the perfusion fluid. The results are recorded in table 2.

Under these experimental conditions no difference was noticed in the glucose and sulphanilamide transport.

Table I

Absorption rates in 1 og glucose nd sulphanilamide in the presence and absence of calcium-binding substances. I all experiments the initial amount of glucose was 30 mg, that f sulphanilamide 3 mg, and the nitial volume was 30 ml. The first and second bsorption periods lasted 20 min tes, and the interval between the two periods was 10 min.

Composition of perfusion fluid in periods 1 and 2	Conc. mM	N mber of experiments	mg glucose bsorbed		‾b 100	mg sulphanilamide absorbed		‾b 100
			First period ( )	Second period (b)		First period ( )	Second period (b)	
1 Sodi m chloride 2 Tetracemin	10	12	22.5	19 7	88	0 91	0.86	91.05
1 Calcium tetracemin 2 Tetracemin	10	15	22.1	19.9	87 1	0.99	0.91	93.3
1 Sodium chloride 2 Sodium fluoride	10	9	23.8	18.3	77.5	1.00	0.83	82.8
1 Sodium chloride 2 Sodium fluoride	20	9	23 9	14.9	60.3	0.87	0.72	82.6
1 Sodium chloride 2 Sodium oxalate	40	13	20.2	17.3	83.8	0.83	0.75	90.8
1 Calcium tetracemin 2 Calcium tetracemin	10	10	23.5	22.1	93 9	1 12	0.98	87 4
1 Sodium chloride 2 Sodium chloride	0	13	22.9	22.5	96.4	0.90	0.78	87.2

Table 2

Rates of absorption of glucose and sulphanilamide *in vivo* from calcium-free and calcium-containing saline. One absorption period of 20 min. duration. Initial amount of glucose 30 mg and of sulphanilamide 3 mg.

Composition of perfusion fluid	Number of animals	mg absorbed	
		glucose	sulphanilamide
30 ml 0.9 % NaCl with 1 mg glucose/ml without Ca	8	22.29 ± 0.77	0.93 ± 0.067
The same saline with a Ca-conc. of 5 mM	8	22.95 ± 0.28	0.99 ± 0.04

Other calcium concentrations have likewise been found in exploratory experiments to have no effect on the same functions.

Water transport from the perfusion fluid was here determined by direct measurement of the fluid volume left in the perfusion vessel and intestine at the end of the absorption period. The effect of sodium oxalate on the net water transport is shown in table 3

Table 3

Absorption of water in millilitres from perfusion fluid influenced by sodium oxalate. Absorption period 30 min.

10 mM sodium oxalate		10 mM sodium chloride	
−1.2		4.8	
−0.2		4.3	
+1.6	Mean + 0.93	3.3	Mean + 3.5
+1.8		3.3	
+1.8		3.8	
+1.8			

A similar effect of sodium oxalate, sodium fluoride and tetracemin on the water transport was seen in exploratory experiments (SÖGNEN unpublished)

It is seen from fig. 3 that, even after an absorption period of 20 minutes, absorption will take place from the perfusion fluid during the next 10 min. at least. This is evidence to suggest that absorption periods of 20 min are suitable in experiments of this kind. Shorter perfusion periods can doubtless likewise be used whereas longer periods are held to be unfavourable.

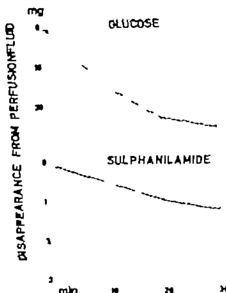


Fig. 3 Amounts of glucose and sulphanilamide disappearing from the perfusion fluid after different absorption periods. Initial amount of glucose 30 mg, of sulphanilamide 3 mg. The tracing is based on at least five observations at each time.

Discussion

As shown by FISHER (1955), there is a relationship *in vitro* between the absorption of urea, creatinine and sorbitol and the transport of glucose and water To discover whether or not the absorption and utilization of glucose have any influence on the absorption of sulphanilamide, the latter has been studied in the presence of glucose.

As seen in fig. 2, the quantity of sulphanilamide transported was slightly reduced when glucose was replaced by equimolar amounts of xylose in the perfusion fluid.

The result of this exploratory experiment suggests that the absorption and utilization of glucose may favour the absorption and utilization of sulphanilamide. It was therefore thought appropriate to study the effect of Ca-b.s. on glucose and sulphanilamide absorption under such experimental conditions as left the absorbing area and hydrostatic pressure approximately unchanged during the absorption period.

As shown in table 1 23 mg glucose disappeared from the perfusion fluid in the course of a 20-min perfusion period. During the second perfusion period the amount of glucose disappearing was 94–96 % of that in the first period. JARVIS *et al.* (1956), in experiments based on the same method, obtained results fairly close to these. With 50 ml of per

fusion fluid containing 1 mg/ml glucose and perfusion periods of 15 min., 19.5 mg glucose were absorbed.

As, however the volume of the perfusion fluid as well as the perfusion period differ in these experiments from ours, the results are not directly comparable.

The main difference between the experiments of JERVIS *et al* and ours lies in the anaesthetic used, pentobarbital sodium in the former and urethane in the latter WILBRANDT & LASZT (1933) have shown that urethane does not affect the absorption of glucose or xylose from isolated loops even at doses of 4500 mg/kg (VERZAR & McDOUGALL 1936). On the other hand urethane is known to affect the permeability of the red blood corpuscles (WILBRANDT 1960) Although the results of the urethane experiments from 1936 must be accepted with some reserve, urethane is in fact an anaesthetic that is used in modern absorption research, e.g. by FOLDVARI *et al* (1962) who employed methods of the same type as those reported here.

The use of air from a tank oxygenator in the gas-lift of the perfusion system proved in exploratory experiments to give the same results as when oxygen was used in the gas-lift.

CURRAN & SOLOMON's perfusion method (1957) is not based on addition of either oxygen or air to the perfusion fluid. It is to be assumed that adequate oxygenation takes place through the blood under such experimental conditions and that the use of air is not more "unphysiological" than the use of oxygen in the gas-lift.

Another difference between JERVIS *et al* s experiments and ours is that the latter had sulphanilamide in the perfusion fluid. However as shown in exploratory experiments, the glucose absorption rate does not depend significantly on the presence or absence of sulphanilamide in the perfusion fluid

The normal blood glucose level in our rats (unfasting) is 113 mg/100 ml (DYBING & SÖGNEN 1958) With 100 mg glucose/100 ml of perfusion fluid the active component of the glucose absorbed should therefore be engaged in possible transport. We must here bear in mind, however that a certain proportion of glucose is utilized, accordingly not proceeding further into the organism (SMYTH 1962) If by absorption from the intestinal tract we understand transepithelial transfer of substance to the extracellular fluid (FISHER & PARSONS 1949), only part of the amount of glucose disappearing can be said to have been absorbed. For utilization to take place, the luminal border of the epithelial cell membrane must also be passed however As shown by NEWEY PARSONS & SMYTH (1959), phlorhizin interferes with the absorption and utilization of glucose by inhibiting the transport of glucose through the luminal border Glucose

that has disappeared from the perfusion fluid will thus in any event have passed the luminal border of the mucosal cells.

There is no significant difference between the absorption of glucose and that of sulphanilamide in the presence or absence of calcium in the perfusion fluid (table 2). However as claimed by DUMONT *et al* (1960) it is impossible in such circumstances to remove all calcium from the perfusion fluid. By this method we must consider calcium to be supplied to the absorbing structure *via* the blood and also to the lumen by secretion and desquamation of epithelial cells. The quantities of calcium thus supplied could suffice to maintain processes that might depend on ionized calcium under such experimental conditions.

In experiments conducted almost simultaneously with these, DUMONT *et al* (1960) showed that the transport of both sodium and water from perfused small intestine loops from the rat can be affected by alterations in calcium concentration. By raising the calcium concentration to 1 mM these workers obtained an increase in water and sodium transport from the intestine. Additional rises in calcium concentration to 5 and 25 mM caused the absorption of water and sodium to fall There seemed, in other words, to be an optimum calcium concentration in their experiments. Glucose was not used there in the perfusion fluid. The hydrostatic pressure was no more than 5 cm, and circulation was established by means of a peristalsis pump, which gave a circulation rate of 0.5 ml/min. DUMONT *et al*'s method seems to be far more useful than that employed in our experiments. Even small variations in water absorption can be recorded and reproduced, whereas this is impossible using SHEFF & SMYTH's method It would be interesting to ascertain whether or not DUMONT *et al.*'s results are reproducible with glucose in the perfusion fluid and whether or not variations in the calcium concentration would influence glucose absorption under their experimental conditions.

Employing sodium fluoride or sodium oxalate in a perfusion system, it will in any event be desirable to work with an approximately calcium-free perfusion fluid, as precipitation of the respective calcium salts will otherwise be unavoidable A continuous recirculation of such a suspension might possibly bring about mechanical action. In our experiments it is therefore assumed that calcium to be bound should be supplied during perfusion and be bound not in the lumen alone, but also in the structures where the conditions are suitable for such binding.

Water transport

An unabsorbable reference standard solution is usually employed for determining water transport in preparations of this kind. CURRAN & SOLOMON (1957) and FOLDVARI *et al* (1962) used human haemoglobin

JERVIS *et al* (1956) with this perfusion method, used sodium thiosulphate as reference standard solution. They found, however the individual variations of water transport to be large in proportion to the quantity of fluid absorbed These workers hold that a perfusion period of less than one hour will not give useful results and that even with one hour perfusion periods only pronounced effects on water transport will be recordable.

An essential cause of the difficulty of studying water transport by this method is perhaps, the preceding washing of the intestine, which probably involves a greatly varying absorption of water during the washing.

As the effect on the water transport in loops *in situ* is unquestionable, only a few characteristic experiments have been included here, with sodium oxalate as absorption depressor The water transport was examined by direct measurement of the volume left after the perfusion period. The net transport of water from the perfusion fluid was seen to be markedly reduced in the presence of sodium oxalate (table 3)

Sulphanilamide transport

Ca-b.s. do not affect the transport of sulphanilamide from the perfused isolated small intestine of the rat under the experimental conditions considered here. When, however sodium fluoride is added to the perfusion fluid at such concentration that morphological changes take place, a reduced rate of disappearance of sulphanilamide is seen. This indicates that damage to the intestinal mucosa impaires sulphanilamide absorption

In the control material, when Ca b s were used in none of the perfusion periods, the sulphanilamide absorption was found to be 10–12 / lower during the second period than during the first. Exploratory experiments showed that only small quantities (about 3 /) of sulphanilamide are transported back to the intestinal lumen during the resting period and during the second period, but these will appear as unabsorbed sulphanilamide in the second period.

An important cause of the difference in sulphanilamide absorption between the first and the second period is presumably the general fall in absorbing capacity manifesting itself by the reduction in glucose absorption both in SHEFF & SMYTH s experiments and in ours. A similar reduced absorption during the second period is also seen for amino acids (JERVIS & SMYTH 1959) and fatty acids (BARRY & SMYTH 1960).

During the second perfusion p[illegible] much [illegible]ilamide was present in the blood that the [illegible] the intestinal lumen and the blood w[illegible] period. Exploratory test have shown tl[illegible]

amide does not reach such a high level as to give an equilibrium of sulphanilamide between the intestine and the blood under these experimental conditions. Contributory causes of this are also, among other things, the renal excretion and the plasma binding of sulphanilamide.

It is seen from fig. 3 that 20-min. perfusion periods are suitable for determining the amount of glucose as well as of sulphanilamide absorbed at a time when the absorption has not yet been concluded.

Exploratory experiments with determinations of sulphanilamide concentration in the perfusion vessel 5, 10, 15, 20, and 30 min. after beginning the perfusion showed the sulphanilamide concentration to be falling during the perfusion period. Sulphanilamide is, in other words, absorbed at a faster rate than water and, at least in part, independent of this.

Sulphanilamide transport will therefore not be affected directly by a reduction in water transport.

Summary

Whereas glucose transport is slightly reduced in presence of Ca b.s. in the perfusion fluid, sulphanilamide transport is reduced only at concentrations of sodium fluoride that cause morphological changes. Water transport is reduced in presence of Ca-b.s. but this does not seem to affect sulphanilamide transport. Water transport was not determined in the initial experiment with xylose in the perfusion fluid. Even if an inhibition of water transport took place then, it would not be justifiable to associate the slight reduction in sulphanilamide absorption with alterations in water transport. The experiments described here thus provide no basis for an explanation of reduced sulphanilamide transport in presence of xylose.

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Intestinal Transport of Glucose and Sulphanilamide In Vitro at Low Calcium Concentrations and in Presence of Calcium-Binding Substances

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(Received August 3 1964)

It has been shown in preceding papers how calcium-binding substances (Ca-b.s.) act on total absorption (SÖGNEN 1961 & 1965 a), gastric emptying time and transit (SÖGNEN 1965 d) in the intact animal, as well as on absorption from isolated and perfused intestinal loops *in situ* (SÖGNEN 1965 c & e). Further it has been shown how the renal excretion of sulphanilamide and urine flow behave in the presence of Ca-b.s. (SÖGNEN 1965 b).

In none of the experiments previously described has it been possible to study the transepithelial translocation alone without considering alterations in blood circulation and other factors that may vary in the intact animal or *in situ*.

In our work the transport *in vitro* of glucose and a sulphonamide has therefore been investigated by WILSON & WISEMAN's method (1954) both in calcium-free Ringer solution and in the presence of Ca-b.s.

Material and Methods

Male rats weighing about 200 g were killed by a blow on the neck. The abdomen and the thorax were opened, but the diaphragm was left intact. The thoracic vessels were cut. Bleeding took place through the thoracic cavity and the nose.

The intestine from the duodenojejunal flexure to the caecum was washed with a 0.9 sodium chloride solution. It was removed quickly from the mesentery in the direction from the caecum and forwards, transferred to a channel containing a 0.9 / sodium chloride solution and everted in this over a glass rod.

Four segments were taken from the oral end of the intestinal loop. The specimens were about 5 cm long and prepared in rapid succession. They were incubated at

intervals of 4 mm. in 150 ml Erlenmeyer flasks containing 30 ml Krebs-Ringer solution.

Before incubation each segment was weighed and filled with 1 ml of fluid, composed as indicated, which had been gassed with a mixture of 95 % O_2 and 5 % CO_2 for 4 min. After incubation for 1 hour the specimens were weighed before and after emptying, and the glucose and sulphanilamide concentrations were determined in the mucosal (outer) and serosal (inner) fluids.

The sulphanilamide determinations were carried out by KING & AL's modification of BRATTON & MARSHALL's method (1939). For the glucose determinations we used HALSE's modification (unpublished) of PROKHOVNIK & NELSON's method (1953).

Solutions

Krebs-Ringer solution with bicarbonate buffer was prepared as described by UMBREIT, BURRIS & STAUFFER (1951). The solution was used partly with and partly without calcium, as described below.

As in repeated experiments the mucous membrane disintegrated in the presence of Ca-b.s. in the incubation fluid, the loops were exposed to Ca-b.s. for a further specified number of seconds before the incubation. To calcium-free Ringer solution was added sodium fluoride, sodium oxalate or neutralized *tetracemin in the concentrations indicated in the tables. The intestinal loops were immersed in these solutions immediately before the incubation.

Results

Fig. 1 illustrates the difference in principle between the transport of glucose and that of sulphanilamide *in vitro*. In the experiments the initial concentrations of glucose and sulphanilamide were the same in mucosal and the serosal fluid. It is seen that the concentration of glucose increased in the serosal fluid after incubation, whereas that of sulphanilamide did not. The results show an active transport of glucose and a passive equilibrium of sulphanilamide between the mucosal and the serosal fluid.

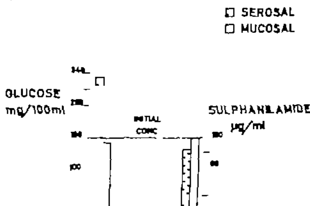


Fig. 1. Main difference between glucose and sulphanilamide transports in everted loops of small intestine. Period of incubation 1 hour. Serosal volume 1 ml, mucosal volume 30 ml. The columns give final concentrations in serosal and mucosal fluid. Mean values of 12 experiments in each group.

* Tetracemin (NFN) Edetic acid (BAN) Edathamil (NNR)

At the beginning of the experiment the glucose concentration was 180 mg/100 ml in the mucosal and the serosal fluid and that of sulphanilamide 100 µg/ml on both sides. Fig. 1 shows that the glucose concentration in the mucosal fluid had fallen to 167 mg/100 ml (s.e.m. ± 4.8), whereas that in the serosal fluid had risen from 180 to 330 mg/100 ml (s.e.m. ± 14.9).

The sulphanilamide concentration was unchanged, 99.3 µg/ml (s.e.m. ± 1.26), in the mucosal fluid, whereas in the serosal fluid it had fallen somewhat, to 84.2 µg/ml (s.e.m. ± 0.82). The fall may be due to uptake of sulphanilamide by the intestinal wall.

Fig. 2 illustrates the transport of glucose and sulphanilamide in the presence or absence of calcium. Sulphanilamide was here added only to the mucosal fluid, the initial concentration in the serosal fluid thus being zero. The glucose transport was studied in the same way as in the preceding experiment.

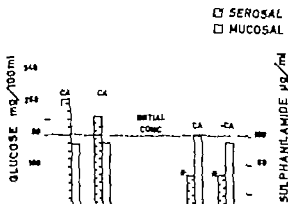


Fig. 2. Transport of glucose and sulphanilamide in everted loops of rat small intestine in the presence and absence of calcium. The columns give the mean values of final concentrations in 12 experiments in each group. Period of incubation 1 hour. Length of loop 5 cm. Serosal volume 1 ml. Mucosal volume 30 ml.
Initial serosal concentration of sulphanilamide = 0.

The final concentration of glucose in the mucosal fluid is in the presence of calcium (155.5 mg/100 ml (s.e.m. ± 14.8) and in its absence (161.5 mg/100 ml (s.e.m. ± 13.1)).

The concentration difference in the serosal fluid indicates a reduction in active transport. In the presence of calcium the final concentration was 269.7 mg/100 ml (s.e.m. ± 13.9) and in its absence 226.6 mg/100 ml (s.e.m. ± 12.4).

The sulphanilamide concentration which initially was 100 µg/ml in the mucosal and nil in the serosal fluid seemed to be equal in the serosal

fluid of experimental and control loops at the end of the experiment. The concentration was 45 8 μg/ml (s.e.m. ± 1 8) in the presence of calcium and 47 4 μg/ml (s.e.m. ± 1 2) in its absence.

The sulphanilamide concentrations in the mucosal fluid likewise did not differ significantly being 101 9 mg/100 ml (s.e.m. ± 1.5) in Krebs-Ringer solution with calcium and 97.2 mg/100 ml (s.e.m. ± 2.5) without it.

The amount of glucose transported was thus somewhat larger in the presence than in the absence of calcium, whereas no difference was demonstrable in sulphanilamide transport.

On incubation of intestinal loops in 1 mM Ca-b.s. solutions, the active transport of glucose was seen to have ceased, but at the same time a marked disintegration of the mucous membrane was noticed (ERICHSEN & SÖGNEN unpublished) This did not show itself by the incubation fluid becoming turbid, but was plainly visible when the intestinal loop was laid on filter paper a few seconds before weighing. It then left a streak of mucus and desquamated epithelial cells on the filter paper

In experiments whose results are recorded in table 1 the intestinal loop was immersed in a 1 mM solution of the Ca b.s. for 30 seconds before the incubation.

At shorter immersion periods (5 and 10 seconds) neither reduction in active transport nor morphological change was observed

Table 1

Effects of calcium-binding substances glucose d sulphanilamide transports in everted loops of rat small intestine.

The preparations were immersed in 1 mM solutions of tetracemin, sodium fluoride or sodium oxalate for 30 seconds before 1 hour of incubation in calcium-free Krebs-Ringer solution.

Initial mucosal and serosal concentration f glucose 180 mg/100 ml.

Initial mucosal sulphanilamide concentration 100 μg/ml and serosal concentration = 0.

	Number of experiments	Final concentration of			
		Glucose mg/100 ml		S lphanilamide μg/ml	
		Mucosal	Serosal	Mucosal	Serosal
Tetracemin	5	151 ± 12.6	165 ± 3 8	49 ± 1.2	99 ± 0.5
Sodium fluoride	5	226 ± 19 9	169 ± 16.8	51 ± 2.3	98 ± 1
Sodium xalate	5	216 ± 11 4	162 ± 10.8	50 ± 2.2	100 ± 1 1
Control Sodium chloride	1	265	151	52	99

Tetracemin is seen to have completely stopped the active transport. Pronounced morphological changes were then present. Sodium fluoride effected a slight reduction in active transport. The specimens had undergone no morphological changes. Sodium oxalate reduced the active transport somewhat more, when some of the specimens displayed appreciable morphological changes (ERICHSEN & SÖGNEN, unpublished)

In the preceding experiments water transport was reduced to zero owing to the high pressure obtained in the serosal (inner) fluid by filling the loop with 0.2 ml/cm intestine (1 ml in an intestinal loop of 5 cm). Exploratory experiments showed that if the intestinal loop is filled with a smaller volume (e g. 0 15 ml/cm loop), water will be transported to the serosal fluid on incubation in calcium-free Ringer solution. In the presence of Ca-b.s. water transport will cease, and the amount of sulphanilamide in the serosal fluid will be reduced according to the difference between the final volumes in the experimental and the control groups By way of example, 1 6 ml of water and 520 µg sulphanilamide will be transported from a mucosal fluid having an initial volume of 30 ml and a sulphanilamide concentration of 100 µg/ml to a serosal fluid with an initial volume of 10 ml and an initial sulphanilamide concentration of nil. In the presence of sodium fluoride the water transport will be nil or negative and the amount of sulphanilamide transported to the serosal fluid 292 µg/ml Such alterations have only been seen in specimens with pronounced morphological changes. A detailed account is therefore of little value

Discussion

The specimen used is assumed to function in the way described below Fluid and dissolved substances are first absorbed by the epithelial cells from the mucosal fluid This correspondens to the first link of the ordinary physiological absorption process. Fluid and substance are transported further to the subepithelial region. *In vivo* they would have been removed from this *via* blood and lymph, but this is not possible *in vitro* Fluid and substance therefore accumulate in the intestinal wall When no more fluid can accumulate in the intestinal wall, some will pass into the serosal fluid

Should the substances whose transport is to be studied only be present in the mucosal fluid, they will, if transported passively pass to the serosal fluid in accordance with the concentration gradient between serosal and mucosal fluids (BARRY 1960)

Substances that are absorbed actively will be transported, even if the concentration is or becomes higher in the serosal than in the mucosal fluid (WILSON & WISEMAN 1954)

In our experiments sulphanilamide was shown to be transported passively in the specimens when conditions were suitable for active transport of glucose (fig. 1)

As the initial concentrations of sulphanilamide in the serosal and the mucosal fluids were equal (100 µg/ml), a reduction in the concentration must have taken place in the former The net transport of water was nil, owing to the pressure in the isolated intestinal segment. The fall in sulphanilamide concentration of the serosal fluid was therefore most likely due to uptake of sulphanilamide by the intestinal wall from the serosal fluid.

Another factor so far not mentioned in connection with these specimens, is the small remnant of fat left at the site of transition from the mesentery to the intestine, which may contribute towards a larger fat content in the serosal fluid than in the mucosal. The distribution of substances over the serosal and the mucosal fluid may therefore be considered to depend on their fat-solubility

Further it has been shown by WILSON (1953 & 1954) that lactic develops during the transport of glucose, to be accumulated as lactate in the serosal fluid. This environmental difference between the serosal and the mucosal fluids may likewise be considered to affect the distribution of substances between the two fluids especially with pH alterations in the serosal fluid during the incubation, and may manifest itself particularly in relation to transport of substances with pK values close to the *pH of* the environment.

With the large volume (30 ml) of the mucosal and the small volume (1 ml) of the serosal fluid, even small variations in the transport from the mucosal fluid will have large effects in the serosal fluid. The specimens are therefore in general most suitable for studies of intestinal transepithelial passage of substances and fluid. However as shown by HANSEN (1958), tetracemin has a disintegrating effect on intestinal mucosa *in vitro* Our work showed the same to be true of the other Ca-b.s. (The disintegration caused by sodium fluoride was seen at higher concentrations or after a longer time of exposure than employed to achieve the results reported here)

Disintegration of an epithelium to detached cells does not necessarily interfere with all the functions worth studying in the individual cell. *Tetracemin is, in fact,* used in tissue cultures to avoid adhesion of large coherent cell areas to the glass walls. Theoretically the intestinal mucosa might therefore be expected to disintegrate under the action of tetracemin, after which the calcium ions could be added in excess. This would give a purer and more clearly defined preparation for studying the transport and metabolism of the *individual cell than that obtained* by scraping

the mucous membrane. Mucosal scrapings yield impure preparations, which have nevertheless given valuable enzymologic information (SOLS 1956).

A similar removal and replacement of ions has been described for erythrocytes (MAIZEL 1961) and for liver homogenates (HERBERT 1958 MINARD & WAGNER 1958).

The effect of tetracemin on intestinal tissue *in vitro* seems to consist of a well-defined denudation of villi (ERICHSEN & SÖGNEN) Ca b.s. are therefore badly suited to the study of transport through membranes *in vitro* It will at any rate be highly time-consuming to find concentrations that can cause functional changes without their being associated with epithelial disintegration.

Morphologically unchanged mucous membranes may possibly be obtainable from the *in vitro* specimens by administering Ca b.s. to intact animals and preparing the *in vitro* preparations from these by killing them at different intervals after the administration.

Summary

The experiments have shown that sulphanilamide is passively transported in everted loops of small intestine, where the conditions were suitable for active transport of glucose. Glucose transfer was reduced and sulphanilamide transfer was unaltered in the absence of calcium from the incubation fluid.

A simultaneous reduction in water and sulphanilamide transport was seen at concentrations of Ca-b s. causing profound morphological changes in the preparations.

Immersion of the intestinal loop in solutions of Ca-b.s. for 30 seconds before incubation revealed reduction in glucose transport that usually went parallel with the morphological changes by the Ca-b.s.

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Glucose Utilization by Intestinal Tissue in the Presence of Calcium-Binding Substances

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Various workers have demonstrated a correlation between the *in vitro* transport of fluid and electrolytes and the presence of glucose (FISHER 1954 SMYTH & TAYLOR 1954 LIFSON & PARSONS 1957 PARSONS & WINDGATE 1961).

We can calculate the utilization of glucose and simultaneously study transport by WILSON & WISEMAN's method (1954), but the results will be of limited accuracy Matthews (personal communication) therefore prefers to study glucose metabolism separately This has also been done in the experiments reported below

Material and Methods

Non-fasting male rats, ranging in weight from 150 to 200 g, were anaesthetized with urethane i.p. The abdomen was opened 10 minutes later and the small intestine segment from the duodenojejunal flexure to the caecum was washed, eviscerated and everted over a glass rod, as described by WILSON & WISEMAN (1954). Fluid was removed from the intestinal loop by carefully brushing the mucous membrane, the intestine being held in the vertical position to facilitate running off The loop was ligated at one end and filled with 10 ml of Krebs-Ringer solution. It was then transferred to a flask containing 30 ml of Krebs-Ringer solution, representing mucosal fluid A gas mixture (95 % oxygen and 5 carbon dioxide) was introduced into the flask for 4 minutes before incubation The incubation period was 1 hour in all the experiments.

Immediately after the end of incubation the flask was transferred to a boiling water bath for 3 minutes, to stop glycolysis. The contents of the flask were homogenized in a Waring blendor for 4 minutes. The homogenate was diluted to 200 ml. A measured portion of the diluted homogenate was quickly transferred to a test

tube, and trichloracetic acid was added to precipitate protein. Glucose was then determined as previously described (SÖGNEN 1965 d)

The glucose metabolism was calculated as the difference between the glucose content of intestinal loops plus incubation fluid, boiled and homogenized immediately after preparation, and that of intestinal loops plus fluid after incubation.

In some experiments glucose was determined in incubated homogenized intestines. The results have been included and discussed and are possibly of theoretical interest.

Results

In these experiments whole intestinal loops containing on an average 3 17 mg calcium (SÖGNEN exploratory investigations) were used. It was considered a matter of interest to discover whether utilization of glucose in ordinary Krebs Ringer solution differed from that in calcium-free incubation fluid.

The results are shown in table 1

Table 1

Glucose utilization in everted loops f rat mall intestine in the absence and presence of calcium. Initial amount of glucose 200 mg. Serosal volume 10 ml. Mucosal volume 30 ml of Krebs-Ringer solution.

Krebs-Ringer sol tion	Number of experiments	Mg glucose utilized/g intestine (wet weight) per hour	P
Calcium-free	17	12.12 ± 2.28	> 0.05
Calci m-containing	13	12.03 ± 2.62	

There was clearly no significant difference in glucose utilization between intestinal loops incubated with and without calcium in the incubation fluid. This result justified omission of calcium in the incubation fluid in subsequent experiments, as sodium oxalate and sodium fluoride would cause immediate precipitation of the respective calcium salts in the incubation solution.

The effects of Ca-b.s. are recorded in tables 2 and 3

Sodium fluoride at a concentration of 1 mM is seen to have slightly inhibited the utilization of glucose. At a concentration of 10 mM the inhibition was 85 %. Further it is seen that sodium oxalate inhibited glucose utilization and that the degree of inhibition depended on the concentration. The same was true of tetracemin. Of the latter 1 mM gave approximately the same inhibition as sodium fluoride at the same concentration, whereas at concentrations of 10 mM tetracemin caused much less inhibition than sodium fluoride.

Table 2

Effects of calcium-binding substances on the glucose utilization in everted loops of rat small intestine *in vitro*. Period of incubation 1 hour

Ca-b.s.	mM	Number of animals	mg glucose utilized/gram intestine	
Sodium fluoride	1	9	10.1 ± 2.8	$0.05 < p < 0.1$
	10	10	1.8 ± 2.0	$p < 0.01$
Sodium oxalate	10	12	10.6 ± 2.1	$0.05 < p < 0.1$
	20	5	8.9 ± 1.7	$0.001 < p < 0.01$
Controls Sodium chloride		17	12.1 ± 2.4	

Table 3

Glucose utilization in solutions of equimolar concentrations of tetracemin and calcium tetracemin. Period of incubation 1 hour

	mM	Number of animals	Mg glucose utilized/gram intestine mean ± s.e.m.	
Tetracemin	1	5	10.1 ± 1.1	$0.1 < p < 0.2$
Calcium tetracemin	1	5	12.1 ± 2.3	
Tetracemin	10	9	9.6 ± 2.1	$p < 0.001$
Calcium tetracemin	10	10	14.2 ± 0.8	

The utilization of glucose in intestinal loops incubated with calcium tetracemin was greater in these experiments than in calcium-free Krebs-Ringer solution.

Little importance can be attached to the difference in glucose utilization in the presence and absence of calcium tetracemin, unless the experiments are performed on the same day.

The mean value is based on the 17 control experiments conducted for the comparison in general and carried out on different days.

Experiments have been conducted with homogenates of intestinal wall in order to arrive at an impression of glucose utilization in intestinal tissue.

Repeated experiments revealed no utilization of glucose in such homogenates.

Discussion

The correlation between the presence of glucose in the medium and the transport of electrolytes and fluid *in vitro* is mentioned in the introduction. Most workers regard this correlation as an indication that the

Tetraceminum (NFN) Edetic acid (BAN) Edathamil (NNR)

glucose forms part of the energy-creating process underlying the active components of the transport system (SMYTH 1962)

Our experiments have given the results that Ca b.s., which have been shown previously to influence water transport, also have a certain inhibitory effect on the utilization of glucose *in vitro*

The effect on glucose metabolism is so relatively small that we must expect no more than a moderate action on the transport systems depending secondarily on this metabolism.

That some glucose seems to be metabolized in the presence of calcium tetracemin (table 3) is possibly due to the pH being lowered so much in certain structures that the calcium tetracemin complex is dissociated and yields calcium ions to the metabolic process in concentrations optimal for this. Such local pH changes are assumed to occur at the absorbing surface of the intestinal tract (SCHANKER *et al* 1958) They might also conceivably arise in the transport system of the intestinal wall in relation to the transport of glucose, which, according to WILSON (1954), is converted into lactic acid during transport and is present as lactate in the serosal fluid. This hypothesis is weakened by the results of the experiments with calcium-free and calcium-containing Krebs-Ringer solution, which showed the glucose metabolism to be the same with both solutions. The results recorded may on the other hand, be due to the calcium concentration chosen not having been optimal (tabel 1)

Glucose, which is generally utilized in tissue homogenates, was not metabolized in homogenates of intestinal tissue under the experimental conditions employed This observation has been confirmed by NEWEY & SMYTH (1962) on the basis of experiments conducted simultaneously with ours. The cessation of utilization of glucose might, perhaps, be related, at least in part, to the fact that no transport takes place It may however also be thought to be caused by a disorganization of enzymes caused by the homogenization. The present work affords no basis for further discussion on this problem but the observation may possibly constitute a useful starting point for a study of the transport and metabolism of glucose in membranes.

Summary

It has been shown that there was no significant difference between glucose utilization by everted intestinal loops incubated with or without calcium in Krebs-Ringer solution

Calcium-binding substances reduced glucose utilization in everted intestinal loops. The degree of inhibition and glucose utilization depended on the concentration of calcium-binding substances.

An apparent increase in glucose utilization in presence of calcium tetracemin was observed and is discussed.

No glucose utilization took place in homogenates of intestinal tissue.

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From the Department of Pharmacology University of Copenhagen
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The Electrolyte Composition of Granulation Tissue Normal, Scorbutic and Thyroxine-Treated Guinea Pigs

By

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(Received July 28, 1964)

Previous publications from this laboratory have dealt with the electrolyte composition of connective tissue in a variety of circumstances, including acute inflammation and treatment with large doses of ascorbic acid. As a part of these investigations the electrolyte composition of granulation tissue, i.e. regenerative connective tissue, has been examined in the study reported here

Methods

JØRGENSEN (1962b and 1963) examined the effect of ascorbic acid on formation of granulation tissue in open wounds and the effect of thyroxine on wound healing in normal and ascorbic acid deficient guinea pigs. Since tissue remnants from these two studies were available at the time when tissue electrolyte determinations were carried out in this laboratory these have now been analysed for sodium, potassium and chloride.

Experimental groups

A 1) *Control* (control on A2 and A3)
   2) *Scorbutic*
   3) *Scorbutic + C vitamin for 2 days*
B 1) *Control* (control on B2)
   2) *Thyroxine-treated*
C 1) *Scorbutic* (control on C2)
   2) *Scorbutic + thyroxine-treated*

Experimental procedures

Female guinea pigs weighing about 300 g were given a scorbutogenic diet consisting of bran, crushed oats and skim-milk powder (9 5 6), with water ad libitum. To this was added an adequate supply of vitamins other than ascorbic acid. (LINDHOLM & THEIL NIELSEN 1956). Control animals received 50 mg of ascorbic acid dissolved in

0.5 ml of 0.9% NaCl, injected i.p. every other day. Scorbutic animals were given control injections without ascorbic acid. The animals in group A 3 received 50 mg of ascorbic acid on two successive days before being killed. Thyroxine-treated animals received daily injections of 50 µg of sodium L thyroxine suspended in 0.5 ml of 0.9% NaCl. After 8 days, wounds were inflicted on the back and granulation tissue produced by the technique of RUDAS (1960 cf. JØRGENSEN 1962 a). The animals were killed 8 days later and the granulation tissue was removed, weighed, freeze-dried to constant weight, defatted to constant weight and subsequently analysed. The amounts of sodium and potassium were determined with a Beckman DU flame photometer and photomultiplier. Chloride determinations were carried out by automatic potentiometric titration (RADIOMETER's ® titrator) after hydrolysing with 0.6 N KOH and precipitation of the proteins by SOMOGYI's technique (cf. COTLOVE 1962). In separate groups of normal, scorbutic and thyroxine-treated animals, no formation of granulation tissue was induced, but the animals were otherwise carried through the same procedures as described above. At the end of the experiment the plasma concentrations of sodium and chloride were determined.

Results

The contents of sodium, potassium and chloride, expressed as mEq/100 g of the dry fat-free tissue, are summarized in table 1. The Na/Cl-ratios are also indicated. In groups of scorbutic animals a significant increase in the chloride content ($p < 0.001$) and a smaller but also significant increase in the sodium content ($p < 0.005$) were observed. The Na/Cl ratio was lowered thereby significantly ($p < 0.001$). A decrease in the Na/Cl-ratio was also observed in non-scorbutic animals treated with thyroxine ($p < 0.001$). In these animals, however, the decrease was caused by a fall in the sodium content ($p < 0.001$). The potassium content was significantly reduced in scorbutic animals ($p < 0.001$).

Table 1

Relative amounts of sodium, potassium and chloride and the Na/Cl-ratio.

Group	*)	N (mEq/100 g⁺) mean s.e.m.	K (mEq/100 g⁺) mean s.e.m.	Cl (mEq/100 g⁺) mean s.e.m.	Na/Cl mean s.e.m.
A 1 Control	14	44.7 ±0.58	37.5 ±0.77	49.9 ±0.81	0.91 ±0.012
A 2 Scorbutic	14	47.7‡ ±1.10	32.2† ±1.01	58.5† ±1.86	0.82† ±0.016
A 3 Scorbutic + C vitamin 2 days	13	44.3 ±1.44	40.5 ±1.02	47.5 ±1.24	0.93 ±0.012
B 1 Control	15	44.3 ±0.92	38.7 ±0.91	47.4 ±0.70	0.93 ±0.015
B 2 Thyroxine treated	14	39.5 ±0.78	38.3 ±0.68	47.9 ±1.36	0.83† ±0.030
C 1 Scorbutic	14	47.4‖ ±0.56	34.4† ±1.03	60.8† ±0.97	0.78† ±0.012
C 2 Scorbutic + Thyroxine treated	14	46.5 ±1.58	32.9† ±1.21	57.1† ±1.18	0.83† ±0.023

*) = number of animals.
⁺) Fat-free solids.
†) Significantly different from the control groups at $p < 0.001$
‡) Significantly different from the control group at $p < 0.005$.
‖) Significantly different from group A 1 at $p < 0.05$.

Table 2

Concentrations of sodium and chloride in the plasma of 6 control animals, 4 scorbutic and 4 thyroxine-treated animals.

Na (mEq/l)			Cl (mEq/l)		
Control	Scorbutic	Thyroxine-treated	Control	Scorbutic	Thyroxine-treated
116	119	120	94	95	93
117	120	120	94	96	97
117	120	120	98	102	98
118	121	121	98	104	100
120			100		
122			10[illegible]		

The figures in table 2 demonstrate that deficiency of ascorbic acid or treatment with thyroxine has no effect on the sodium or chloride contents of the plasma (owing to slight haemolysis in the blood samples the potassium values have not been reported)

Discussion

Granulation tissue of scorbutic animals (groups A2, C1 and C2) contains more sodium and chloride than does granulation tissue of control animals (groups A1 and B1) or scorbutic animals treated with vitamin C (group A3) although plasma electrolyte concentrations are the same (table 2) This may in part be due to increased exudation in scurvy which would also contribute towards the occurrence of increased amounts of granulation tissue in these animals (cf JØRGENSEN 1962b).

That a difference in degrees of exudation is not the only factor responsible for the electrolyte changes is, however demonstrated by the changes in the Na/Cl ratio Although increased exudation would tend to raise this ratio it is significantly reduced in scorbutic animals. This increase in the chloride content therefore seems to be of a more specific nature

Ascorbate ions are among the ions that may be bound to macromolecules of connective tissue (ENGEL *et al* 1961) and the *in vivo* studies of LANGGÅRD (1964a & b) have shown that ascorbate ions in normal connective tissue may compete with chloride ions bound to hyaluronic acid. Several authors (ABT *et al* 1959 POSTLETHWAIT *et al* 1960 SCHANBLE *et al* 1960) have further demonstrated a specific accumulation of ascorbic acid in granulation tissue. In view of these findings it seems reasonable to suggest that, whereas ascorbate ions in normal granulation tissue to a great extent occupy the cationic side groups of hyaluronic acid, some of them

are free to bind chloride in the absence of ascorbate ions. Several other factors, moreover may be involved at the same time.

A prominent feature of granulation tissue of scorbutic animals is extravasation of erythrocytes (STOLMAN *et al* 1961 JORGENSEN 1963) It should therefore be noted that the potassium content is significantly reduced in scorbutic animals (table 1) However it should be remembered that the degree of plasma exudation is also greater in scorbutic animals and that dilution of the tissue with plasma represents a relative "dilution" of tissue potassium expressed as mEq per 100 g of the dry fat free tissue. Apparently the dilution effect overcompensates for the increase in potassium caused by the accumulation of erythrocytes in the tissue.

The decrease in the sodium content of thyroxine treated animals may be due to a direct effect of thyroxine on acid mucopolysaccharides of the tissue. MOLTKE (1957) found a reduced incorporation of radioactive sulphate in linear wounds of thyroxine treated animals, and JORGENSEN (1963) by histochemical reactions demonstrated a reduction in the amount of mucopolysaccharides in scorbutic animals treated with thyroxine, compared with the increased amount seen in scorbutic animals. Treatment with thyroxine may thus have a depolymerizing effect on the hyaluronic acid. This would reduce its sodium-binding capacity but would also reduce the capacity for binding potassium (KULONEN 1952) and water (HVIDBERG 1962). Reduced plasma exudation, on the other hand, would also depress the relative sodium content, but would raise the relative potassium and water contents The combined effect of a slight depolymerization of the hyaluronic acid and of a reduction in plasma exudation, can therefore explain both the decrease in sodium and the unchanged potassium and water contents (cf JORGENSEN 1962b).

Summary

Sodium, potassium and chloride determinations have been carried out on granulation tissue of guinea pigs. The groups examined were control animals, scorbutic animals, scorbutic animals treated with ascorbic acid for 2 days, thyroxine-treated animals and scorbutic animals treated with thyroxine.

In scorbutic animals significant increases in the amounts of sodium and chloride were observed. The NaCl-ratio was significantly reduced. The potassium content was significantly reduced After two days of treatment with ascorbic acid these values had returned to normal.

Treatment of normal animals with thyroxine reduced the amount of sodium significantly

Plasma electrolyte concentrations were normal in scorbutic animals and in animals treated with thyroxine.

The results have been interpreted in terms of a specific binding of ascorbic ions to insoluble colloids of the tissues, a depolymerizing effect of thyroxine on hyaluronic acid and varying degrees of plasma exudation.

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The Effect of the Mersalyl-Theophylline Complex on Connective Tissue Electrolytes

By

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(Received July 28, 1964)

The question whether diuretics, besides exerting a direct renal action, have a pre-diuretic effect on the tissues has long been debated. In the present study the problem has been approached by determining simultaneously the amounts of sodium, potassium and chloride in plasma and skin of mice treated with the mersalyl-theophylline complex.

Methods

White male mice of the Leo-strain, weighing from 22 to 27 g, were divided into two groups: one was treated with s.c. oestradiol monobenzoate, 10 µg in 0.1 ml of arachis oil injected 6 and 4 days before the experiment. On the day of the experiment the animals were placed on pieces of filter paper. As spontaneous urination occurred, 0.1 ml of a 1:20 dilution of injectable mersalyl (Ph. Dan. 1948, mersalylum g 100, theophyllinum g 50, 1 N NaOH q.s., sterile water to 1000 ml) in 0.9 / NaCl, i.e. 20 mg of mersalyl per kg body weight, was injected intraperitoneally. Control animals received 0.1 ml of 0.9 / NaCl. Twenty minutes after the injection (the animal did not void during this period), the animal was stunned by a blow on the neck, the urethra was immediately closed by a clamp, and 500 µl of blood were sampled through an incision in the right side of the neck. After beheading and bleeding the animal, the bladder was emptied and the urine measured with a tuberculin syringe. The skin on the back was depilated, and a specific area was marked by a stamp. This area of the skin, with its underlying subcutaneous tissue, was excised and weighed, freeze-dried to constant weight, defatted to constant weight and subsequently analysed. Hexosamine, hydroxyproline, sodium, potassium and chloride determinations on the skin and sodium, potassium and chloride determinations on the plasma were carried out as described by Langgård, Jensen-Holm & Hvidberg (1963).

Results

In table 1 are indicated the amounts of urine produced during the experimental period, the weights of 5.5 cm² of the skin and the relative amounts of water, hexosamine and hydroxyproline expressed as mg or g/100 g of the dry fat free tissue. The plasma and skin contents of sodium,

Table 1

Amount of urine produced in 20 minutes, weight of 5.5 cm^2 of the skin and analytical findings for plasma and skin from oestradiol-treated, mersalyl-treated, (oestradiol + mersalyl)-treated and control animals. Each group represents 15 animals. The mean values are given with their standard errors.

Treatment		*Control*	*Mersalyl*	*Oestradiol*	*Oestradiol + Mersalyl*
Amount of urine/20 min. (µl)		34 ± 8.5	32 ± 11.9	50 ± 8.8	53 ± 14.7
Weight of 5.5 cm^2 (mg)		[illegible]58 ± 11.5	244 ± 8.3	497 ± 13.8	483 ± 18.8
Water content (g/100 g+))		305 ± 6.3	293 ± 9.4	585 ± 16.0	598 ± 26.4
Hexosamine (mg/100 g+))		491 ± 6.8	483 ± 10.9	966 ± 59.5	981 ± 69.3
Hydroxyproline (g/100 g+))		6.8 ± 0.26	7.1 ± 0.09	5.4 ± 0.18	5.6 ± 0.18
Plasma	sodium (mEq/l)	145 ± 1.3	144 ± 1.9	144 ± 1.3	146 ± 1.5
	potassium (mEq/l)	3.9 ± 0.13	3.9 ± 0.14	3.8 ± 0.13	3.6 ± 0.15
	chloride (mEq/l)	104 ± 0.8	102 ± 0.7	104 ± 0.7	105 ± 0.9
Tissue	sodium (mEq/100 g+))	27.9 ± 0.64	27.9 ± 0.78	72.0 ± 3.00	73.3 ± 4.50
	potassium (mEq/100 g+))	19.9 ± 1.44	17.9 ± 0.82	24.8 ± 0.99	22.7 ± 0.63
	chloride (mEq/100 g+))	27.8 ± 0.79	26.2 ± 0.81	62.3 ± 2.14	64.0 ± 2.12

+) Dry fat free tissue.

potassium and chloride are expressed as mEq/l plasma resp. 100 g dry fat free tissue. No differences between mersalyl treated groups and control groups were recorded.

Discussion

Several investigators have presented evidence for a peripheral action of diuretics in mobilizing tissue fluid and electrolytes before the onset of diuresis. The problem has been investigated by studying local phenomena, such as the flow of fluid through subcutaneously inserted canulas, the spreading reaction and the rate of subcutaneous absorption. Of more interest, however, are studies based on blood analyses. CRAWFORD & MCINTOSH (1925) and SERBY (1926) after administration of novasurol ® to oedematous patients found initial hydraemia. Initial hydraemia and increased plasma chloride concentration were also observed by MØLLER (1930) after injecting mersalyl into nephrectomized rabbits. An increase in plasma volume after treatment with diuretics has also been reported (DECHERD *et al.* 1944, RIEZEN 1964). However, many other investigators have been unable to demonstrate any changes in specific gravity, osmotic pressure or electrolyte concentrations of the blood after treatment with

mercurial diuretics (SCHMITZ 1933 BLUMGART *et al* 1934 BRYAN *et al* 1935 DEVRIES 1946 EDLUND & LINDERHOLM 1949)

Direct analyses on the tissues after systemic treatment with diuretics have been carried out only to a limited extent HVIDBERG, SCHMIDT & SCHOU (1958) and HVIDBERG (1959) found no changes in water and hexosamine contents of the skin of mice treated with the mersalyl-theophylline complex. Observations on the effect of diuretics on the electrolyte composition of connective tissue have, as far as we know not been reported previously although such information seems to be highly relevant The entirely negative results obtained in our study although limited to a single observation period, lend no support to the idea of a pre-diuretic peripheral action of diuretics. Electrolyte determinations were carried out on plasma and skin of mice pretreated with oestradiol as well as of normal mice. Treatment with oestradiol in this specific strain of mice causes a considerable increase in the amount of acid mucopolysaccharides of the skin (review HVIDBERG, SZPORNY & LANGGÅRD 1963). The capacity of the skin to serve as a "reservoir" for water and electrolytes is thereby greatly increased (HVIDBERG, JENSEN-HOLM & LANGGÅRD 1963 LANGGÅRD 1964a LANGGÅRD 1964b), and an initial shift of water and electrolytes from tissue to plasma, resulting from treatment with diuretics, might therefore have been easier to demonstrate in these animals (cf LANGGÅRD JENSEN-HOLM & HVIDBERG 1963 LANGGÅRD 1964c). Thus, the fact that no changes were observed either in this group throw further doubt on the concept of an "extra-renal" effect.

Summary

The effect of a combined mersalyl-theophylline preparation on the electrolyte composition of plasma and skin has been examined in normal and oestradiol-treated mice before the onset of diuresis. The results gave no support to the concept of a pre-diuretic peripheral effect of diuretics.

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Interfering Substances by Determination of Poisons in Autopsy Material III
p-Hydroxyphenylacetic Acid, *p*-Hydroxybenzoic Acid and *p*-Hydroxybenzaldehyde

By

Bent Kempe

(Received August, 27 1964)

Continuing previous investigations into impurities that may interfere with spectrophotometric analyses of more or less putrified autopsy material for barbituric acids we have examined further three phenolic compounds *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and p-hydroxybenzaldehyde. A description is given below of the isolation and identification of the three compounds and methods are indicated for separating them from 5,5-substituted barbituric acids.

Methods

General technique

Sublimation, recording of spectra and determination of such constants as melting point, distribution coefficients and R_F values were performed by the previously described technique (KEMPE 1964). Three human livers were used for analysis.

A. *p-hydroxyphenylacetic acid.*

The liver used for extraction had been stored in the minced state for 9 months at 4–5°. The liver had been obtained on medico-legal autopsy of a 73-year old infirm man. There is no reason to suspect him of having consumed any appreciable amounts of poisons. The liver presented no gross signs of putrefaction. It had an amine like odour.

Extract of liver tissue was prepared as follows

Liver tissue, 20 g, was homogenized with 3 ml of 8 N-H_2SO_4 in an M.S.E. homogenizer. The homogenate was extracted three times, each time with 50 ml of ether. The

combined ether extracts were dried with anhydrous sodium sulphate, filtered and evaporated on steam bath to 4–5 ml. The residue was heated three times on a boiling water bath, each time with 10 ml of 0.1 N-H_2SO_4. Subsequent boiling for about 1 minute was succeeded by cooling under the tap to about 20° and filtration. The combined filtrates were extracted with an equal volume of chloroform, which was rejected. To the filtrate were added 2 N NaOH to pH 10–11. Another extract with an equal volume of chloroform was also rejected. After addition of 8 N-H_2SO_4 to pH 1–2 three further extracts, each with an equal volume of ether were combined, dried, filtered and evaporated to dryness on a steam bath. The residue was submitted to chromatographic purification on Whatman paper no. 1 being developed with ALGERI & WALKER's (1952) mixture. Observation at wavelength 254 mμ after spraying with 0.1 N-NaOH revealed two dark spots having R_F values 0.05 and 0.16. Succinic acid and phenyl acetic acid would, if present, have R_F values of 0.03 and 0.34, respectively. The areas covering the stated dark spots were cut out and eluted with 3 ml of 0.1 N-NaOH. On measurement in ultraviolet light, the eluate of the specimen containing the spot with R_F value 0.16 showed an absorption curve of the same appearance as that of phenol under identical conditions. The absorption curve for the other eluate had its maximum and minimum displaced towards a lower wavelength. To the former of these two eluates was added 2 N-H_2SO_4 to pH 1–2. The mixture was extracted three times, each time with three times its volume of ether. The combined dried and filtered ether extracts were evaporated on a water bath. The residue was sublimed at a temperature of 114° and a pressure of 5 mm Hg. The sublimate, judged to weigh about 0.7 mg, consisted of blunt white crystals.

B. *p-hydroxybenzoic acid.*

As the amount of substance in the eluate of the specimen containing the spot with R_F value 0.05 (see preceding section) was judged to be too small for identification, the extraction was repeated. As control we used another liver which had been stored in the minced state for 3 years. The original forensic chemical analysis in this Department had disclosed no poisons of any kind in this liver. It presented no gross signs of putrefaction but also had an amine like odour.

An extract of the liver was prepared as described below.

As described above, 50 g liver tissue, 20 ml of 96% ethanol and 5 ml of 8 N-H_2SO_4 were mixed by homogenisation. The homogenate was extracted three times, each time with three times its volume of ether. The combined ether extracts were extracted once with 20 ml of 0.1 N H_2SO_4, which was rejected. Then followed extraction three times, each time with about 30 ml of 0.1 N NaOH. The combined 0.1 N-NaOH extracts were acidified with 8 N H_2SO_4, and extraction was performed four times, each time with an equal volume of ether. The combined ether extracts were dried with anhydrous sodium sulphate, filtered and evaporated on a steam bath to 4–5 ml. The residue was heated three times on a boiling water bath, each time with 50 ml of 0.1 N-H_2SO_4. After boiling for 1 min. the 0.1 N-H_2SO_4 extract was cooled to about 20° under the tap and filtered. The combined filtrates were extracted three times, each time with an equal volume of ether. The dried and filtered ether extracts were evaporated on a steam bath to 4–5 ml. The remaining ether was removed by standing at about 25°. The residue was submitted to purification by paper chromatography as described above. The area containing the spot with R_F value 0.05 was cut out and eluted with about 10 ml of 0.1 N-NaOH. To the eluate were added about 3 ml of 2 N-H_2SO_4 to give an acid reaction. It was extracted three times, each time with three times its volume of ether. The combined dried and filtered ether extracts were con-

tiously evaporated to dryness, as indicated above. The residue was purified by sublimation at a somewhat higher temperature than that of the previous sublimation. The sublimate, judged to weigh about 1.5 mg, consisted of compact slightly yellow crystals.

C. *p-hydroxybenzaldehyde.*

The liver used for extraction of this substance had been stored in the minced state for about one month at 4–5°. The original forensic chemical analysis in this Department had disclosed no poisons of any kind, except traces of phenobarbitone (the patient had been an epileptic). Death had probably been caused by renal disease.

The liver displayed no signs of putrefaction, but had a disagreeable odour.

An extract of the liver was prepared as described below.

After adding 8 N H_2SO_4 to give an acid reaction 100 g minced liver tissue were homogenised. The homogenate was extracted three times, each time with twice its volume of ether. The combined ether extracts were extracted twice, each time with 40 ml of N-NaOH. The combined NaOH extracts were acidified with 8 N-H_2SO_4.

After extraction three times, each time with an equal volume of ether the combined ether extracts were *dried with anhydrous sodium sulphate, filtered and evaporated* to 15 ml on a steam bath. The residue was heated three times on a boiling water bath, each time with 40 ml of 0.1 N-H_2SO_4. The cooled aqueous extracts were filtered and *extracted three times at pH 6.3 each time with an equal volume of chloroform.* The insoluble residue left after boiling with 0.1 N H_2SO_4 was boiled once more with the extracted aqueous phase of pH 6.3, filtered and extracted with an equal volume of chloroform. The extract was added to the other three chloroform extracts. The combined dried chloroform extracts were evaporated to 20 ml on a water bath. The rest of the chloroform was removed in a current of air.

The residue was purified by paper chromatography as described by ALGERI & WALKER's (1952) system. The developed and dried chromatogram was observed at wavelength 254 mμ. Two dark spots were seen with R_F values 0.45 and 0.55 (phenobarbitone, which, as already stated, had been demonstrated previously). The area covering the spot with R_F value 0.45 was cut out and eluted with 20 ml of 0.1 N borate solution at pH 10.8. The eluate was extracted with ether at acid reaction. The combined dried ether extracts were cautiously evaporated to dryness. The residue was sublimed. The sublimate was collected on the outside of the bottom of a 15 ml flat bottomed tube filled with cold water instead of on the glass-slide used by EDER (1912), at a temperature of 125° and atmospheric pressure. The sublimate, judged to weigh about 0.3 mg, consisted of white crystals.

Results

The crystalline substance isolated by *extraction A* had melting point 147° (corrected). Its infrared and ultraviolet spectra were recorded (see fig. 1 and 2). The curves for the ultraviolet range are practically identical with those for phenol.

The constants found accord closely with the data given in the literature for *p*-hydroxyphenylacetic acid. As material for comparison we used a purchased sample of *p*-hydroxyphenylacetic acid purum Fluka. The sample had melting point 148 (corr.) and an equivalent weight of 151.9

650 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 cm⁻¹

Fig. 1 Infrared spectra of the substance isolated (upper curve) and p-hydroxyphenylacetic acid (lower curve) i KBr

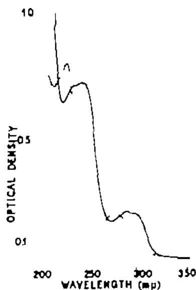


Fig. 2. Ultraviolet spectra of the substance isolated *in 0.1 N borate* buffer pH 10.6 ——— and n 0.1 N HCl ———

calculated from titration with NaOH. Its infrared and ultraviolet spectra were recorded. Its R_F value on paper chromatogram, developed with ALGERI & WALKER's (1952) mixture, was 0 16.

The mixed melting point for a mixture with the substance isolated from the liver was found to be 148 (corr)

The melting point of the crystalline substance isolated by *extraction B* was 207° (corr) The infrared and ultraviolet spectra of the substance are shown in figs. 3 and 4

The constants found correspond closely with the data given in the literature for p-hydroxybenzoic acid. As material for comparison we

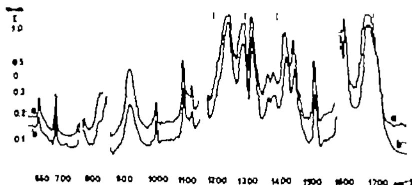


Fig 3 Infrared spectra of the substance isolated (curve b) and p-hydroxybenzoic acid (curve) in KBr

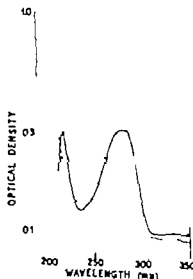


Fig. 4. Ultraviolet spectra of the substance isolated in 0.1 N NaOH ——— and in 0.1 N-HCl ———

prepared *p*-hydroxybenzoic acid by saponifying the methyl ester using the substance indicated in Ph.Dan. 1948 The acid was isolated by extraction with ether at acid reaction The ether was dried and distilled off. The residue consisted of white crystals with an equivalent weight of 137 6 calculated from titration with NaOH A measured portion of the residue was used for sublimation The sublimate had melting point 211 (corr) Its infrared and ultraviolet spectra were recorded. Paper chromatography of the p-oxybenzoic acid prepared, as described by ALGERI & WALKER (1952), gave an R_F value of 0 05 A mixture with the substance isolated from the liver was found to have melting point 211 (corr).

The infrared and ultraviolet spectra of the substance isolated by *extraction C* were recorded, as shown in figs. 5 and 6

The spectra found correspond closely with the data given in the literature for *p*-hydroxybenzaldehyde. As material for comparison we used a purchased sample of *p*-hydroxybenzaldehyde purum Fluka, melting point 116 (corr) Its infrared and ultraviolet spectra were recorded. Its R_F value, measured by paper chromatography as described by Algeri & Walker was 0 45

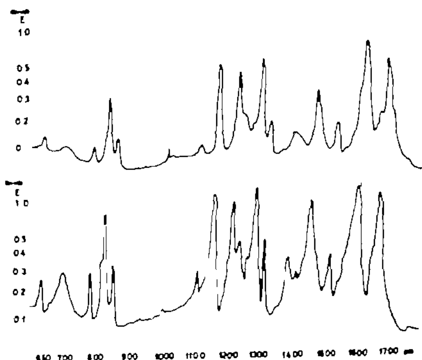


Fig. 5. Infrared spectra of the substance isolated (upper curve) and p-hydroxybenzaldehyde (lower curve) i KBr

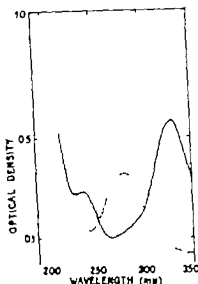


Fig. 6. Ultraviolet spectra of the substance isolated in 0.1 N borate buffer pH 10.8 ——— and in N HCl ———

On comparing the values obtained for the crystalline substances extracted from livers with those for the purchased *p*-hydroxyphenyl acetic acid, the prepared p-hydroxybenzoic acid and the purchased *p*-hydroxybenzaldehyde we found the three isolated crystalline substances to be identical.

Spectrophotometric measurements of *p*-hydroxyphenyl acetic acid and p-hydroxybenzaldehyde in aqueous solution at both acid (pH 2) and alkaline (pH 10) reaction have shown the same conditions of absorption for these substances as for 5,5-substituted barbituric acids at wavelengths near 240 mμ. We therefore attempted to separate the former compounds from barbituric acids. Maxima and minima of the absorption curves for *p*-hydroxybenzoic acid are, as stated previously displaced towards a lower wavelength, as compared with maxima and minima of the absorption curves for *p*-hydroxyphenyl acetic acid. The substance therefore does not interfere so much as *p*-hydroxyphenyl acetic acid with the spectrophotometric analysis for barbituric acids. However the presence of *p*-hydroxybenzoic acid will more or less distort the shape of the curve for 5,5-substituted barbituric acid derivatives, depending on the quantitative relations. On this account we have also attempted to devise a method of separation.

Separation of p-hydroxyphenyl acetic acid, p-hydroxybenzoic acid and p-hydroxybenzaldehyde from barbituric acid derivatives

The distribution coefficients for *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde are given in table 1

Chromatography of p-hydroxyphenyl acetic acid on a ½ mm thick activated silicagel-G plate developed with ALGERI & WALKER's (1952) mixture revealed a red spot with R_F-value 0.21 after spraying with GERNGROSS *et al* s (1933) reagent. A mercuric sulphate reagent in place of GERNGROSS *et al* s reagent gave no reaction. Our investigations have shown that in analysing material for barbituric acids simultaneous extraction of *p*-hydroxyphenylacetic acid and p-hydroxybenzoic acid can be almost completely avoided by using chloroform for the extraction.

Table 1

Partition coefficients for *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde.

		Aqueous phase/ether	Aqueous phase/chloroform
p-hydroxyphenylacetic acid	0.5 N HCl	about 1.3	almost infinite
	0.1 M phosphat pH 5 1	– 3.2	
	0.1 M phosphate pH 6.8	– 15.2	
p-hydroxybenzoic acid	0.1 N-HCl	– 0.13	almost infinite
p-hydroxybenzaldehyde	0 1 N H_2SO_4	0.08	about 1 47
	0 1 M phosphat pH 6.3	– 0.08	1.21

Separation can also be obtained by intercalating paper or thin-layer chromatographic purification. Further a simple calculation, like that indicated for *p*-hydroxyphenylethanol (KÆMPE 1964) allows us to estimate directly from spectrophotometric measurement the largest amount of barbituric acids that could be present in a solution contaminated with *p*-hydroxyphenylacetic acid.

It is impossible to separate *p*-hydroxybenzaldehyde from 5,5-substituted barbituric acids by extraction. By paper chromatography with ALGERI & WALKER s (1952) mixture, most 5,5-substituted barbituric acids shows higher R_F values than *p*-hydroxybenzaldehyde (R_F = 0.45), except barbitone (R_F = 0.48) It is, however possible to remove *p*-hydroxybenzaldehyde by other means, as it is somewhat volatile. By placing the flask containing the dried residue from the ether extraction in a boiling water bath for 10 minutes we can remove 90 / of the substance, but this method cannot exclude loss of minor amounts of barbituric acid derivatives.

Discussion

As practically no p-hydroxybenzoic acid or p-hydroxyphenylacetic acid is transferred to chloroform by extraction from an equal volume of 0.1 N-HCl, we cannot expect interference with these compounds on spectrophotometric examination for barbituric acids when using chloroform as extracting agent. If we wish to include results for the metabolites of barbituric acids, we must isolate these partly by salting out and partly by means of other extracting agents (e.g., ether). In these circumstances the conditions extraction of p-hydroxyphenylacetic acid and p-hydroxybenzoic acid will change so much that these compounds may appear along with the barbituric acid metabolites. As, moreover the two phenols appear at approx. the same R_F-values as hydroxy-substituted barbituric acid metabolites in several of the ordinarily employed systems of paper chromatography we cannot ignore the possibility of their presence in material being analysed for barbituric acid metabolites. The previously described compound *p*-hydroxyphenylpropionic acid (KAMPE 1964) parallels *p*-hydroxyphenylacetic acid in its extraction and will behave like this during analyses for barbituric acids and their metabolites.

Our investigations have thus shown that the phenol-like absorption curve often seen in the spectrophotometric search for barbiturates after extraction with chloroform cannot be due to p-hydroxyphenylacetic acid or *p*-hydroxyphenylpropionic acid, but must be attributed to other substances e.g. *p*-hydroxyphenylethanol, previously isolated by us (KAMPE 1964)

The shape of the curve for *p*-hydroxybenzaldehyde has been seen previously in this Department, e.g. on examination for warfarin and its metabolites. The conditions of extraction and the curve for p-hydroxybenzaldehyde at alkaline reaction are identical with those for the metabolite of warfarin described by WANNTORP (1960)

On the basis of our results we cannot decide whether *p*-hydroxybenzaldehyde is a laboratory artefact produced by the action of an alkali on *p*-hydroxyphenylpyruvic acid (DOY 1960) or is due to an abnormality of metabolism (DENTON, DUBBETT & WALLS 1963). However on testing for substances of an acid character we have often seen a curve shaped like that recorded when measuring *p*-hydroxybenzaldehyde. Finally it may be mentioned that the R_F value for the impurity described by DENTON *et al* (1963) and the spectrum within the infrared range are, in our view consistent with our findings for *p*-hydroxybenzoic acid, although DENTON *et al* did not identify the substance with this. The compound described by DENTON *et al.* is therefore in all likelihood identical with the *p*-hydroxybenzoic acid we have demonstrated. However attention should

be called to the fact that an ammoniacal solution of p-hydroxybenzoic acid does not have its absorption maximum at the ultraviolet wavelength stated (288 mμ) but at 283 mμ.

Summary

One of the impurities with the "phenol like" absorption curve often seen on spectrophotometric examination for 5 5-substituted barbituric acids has been identified as the phenol *p*-hydroxyphenylacetic acid. Two other impurities, which likewise may interfere with spectrophotometric analyses for barbiturates and their metabolites, have been identified as *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde. Methods are indicated for separating *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde from generally occurring barbituric acid derivatives in forensic chemical examinations.

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Protective Action of some Anaesthetics Against Anoxia

By

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During a study of the influence of barbiturates on the resistance of mice to anoxia, ARNFRED & SECHER (1962) found a longer survival time for anoxic mice anaesthetised with barbiturates than for unanaesthetised mice exposed to the same low oxygen concentrations.

In 1932 QUASTEL & WHEATLEY showed that oxygen uptake becomes reduced during anaesthesia. This is considered to be a result of the anaesthesia and not its cause (JOWETT 1938)

DEUTSCH *et al.* (1962) investigated the effect of halothane on circulation and respiration and found the total oxygen uptake to have been reduced by from 20 to 43 %.

It seems natural to assume that the depressing action of barbiturates on cerebral oxygen consumption of animals anaesthetised with them would cause the animals to be more resistant to anoxia than non-anaesthetised animals. This hypothesis was borne out by the results of ARNFRED & SECHER (1962) Their results prompted us to investigate whether barbiturates alone possess this property or whether it is possessed by anaesthetics in general.

Methods

As experimental animals we used male white mice weighing from 25 to 30 g.

The anaesthetising apparatus employed was a *modification of that devised by* POULSEN & SECHER (1949) (fig. 1).

The gases used are conducted from the steel cylinders (A) through flowmeters to an evaporation chamber in which is a spiral wire wound with two layers of gauze (C). The liquid anaesthetic used is contained in the fixed leg of two connected vessels. This fixed leg communicates above with the *evaporation chamber. The anaesthetic* rests on a mercury column. By raising the other mobile, leg of the two connected

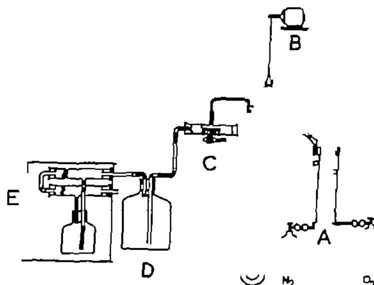


Fig. 1 Experimental mounting of anaesthesia apparatus
A Steel cylinders containing nitrogen and oxygen
B Electromoto
C Evaporation chamber
D Mixing chamber
E Thermostat with bottles for experimental animals

vessels, the anaesthetic will be compressed into the evaporation chamber where it is distributed on the gauze. An electric glow-lamp placed under the evaporation chamber serves to heat this and secure complete evaporation. With a constant gas flow we can calculate how much of a given anaesthetic must evaporate per minute to yield the desired concentration of the anaesthetic in the gas mixture. The mobile leg of the connected vessels is raised by an electric motor (B), whose number of rotations can be varied continuously by means of a gear from 3 to 200 rotations/min. The gas mixture passes from the evaporation chamber through a mixing chamber (D) and from this into five wide-necked bottles (E), each of volume of 300 ml. Each bottle is provided with a double-bored rubber stopper. These five bottles are kept in a thermostat. The working temperature employed was 32–34.

By filling the mixing chamber with smoke the gas mixture was shown to be distributed simultaneously in all five bottles; accordingly any differences of survival among mice cannot be due to delayed onset of anoxia in some bottles.

Analysis in a Bechmann oximeter of the gas mixture conducted to the individual mouse bottles showed that the oxygen concentration was the same in all five bottles and that no difference was noticed in the time elapsing from the reduction of the oxygen supply to the fall of oxygen concentration in the five bottles.

Each experiment was preceded by estimation of the concentrations required of the individual anaesthetic to produce anaesthesia of the animal used ("induction concentration") and to maintain the anaesthesia ("maintenance concentration"). As a criterion of the anaesthesia obtained we employed the abolished response to sound impressions and to painful stimuli. These animals were not used for the final experiments, but were killed after surviving for at least 24 hours.

Five duplicate experiments were carried out with each anaesthetic. Each experiment was on ten mice, two in each bottle a constant flow of air was passed through the bottle, while the mice were being placed in it. After all the mice had been so placed, we started the apparatus. Anaesthetisation was begun with the previously estimated "induction concentration" 20 % oxygen and 80 % nitrogen. When sleep had been induced in all the animals, we continued for 10 to 15 minutes with the "maintenance concentration" still using 20 % oxygen. Then the oxygen concentration was reduced to 5 % and that of nitrogen raised correspondingly the concentration of the anaesthetic remaining unchanged. The survival time, i.e. the interval between the reduction of the oxygen concentration to 5 and end of respiration, was measured for each animal.

After each experiment with a group of ten anaesthetised mice there was a control experiment, constituting the other part of the duplicate experiment, also on ten mice. The mice of the control experiment were exposed to an oxygen-and-nitrogen mixture containing 5 % oxygen. Here, too the survival time was measured.

A flow of 6 litres of gas mixture/min. was employed in all the experiments.

Thus, for each anaesthetic investigated we used 100 mice, of which 50 were anaesthetised, with 50 controls.

The anaesthetics tested were

Thiopentone	= thiomebumal (NFN) = (thiopental (WHO USAN) = leopental ®)
Ether	= aether ad narcosin (NFN)
Chloroform	= chloroformium ad narcosin (NFN)
Trichloroethylene	= trichloraethylenum ad narcosin (NFN) = (trilene ®)
Cyclopropane	= cyklopropan (NFN)
Halothane	= halotan (NFN WHO) (= fluothane ®)
Methoxyflurane	= methoxyfluranum (NFN, WHO) (= penthrane ®)
Nitrous oxide	= nitrogeni monoxidum (NFN)

Results

The "induction concentration" used, the interval until sleep occurred the "maintenance concentration" and the time until anoxia set in are recorded in table 1 the sign / refers to v/v

Table 1

Survey of the concentrations of anaesthetics, to induce the anaesthesia (= induction concentration) and to maintain it (= maintenance concentration), as well as interval from beginning anaesthetisation to occurrence of sleep and transition to the maintenance concentration (= induction time).

Anaesthetic	Induction concentration (%)	Induction time (min.)	Maintenance concentration (/)	Anoxia after (min.)
Ether = aether ad narcosin (NFN)	6	15	4	10
Chloroform = chloroformium ad narcosin (NFN)	2	3	1	10
Trichloroethylene = trichloraethylenum ad narcosin (NFN)	3	5	1.5	10
Cyclopropane [illegible]	30	3	25	10
[illegible]	2	5	1	10
[illegible]	1.5	5	0.5	15
[illegible]	20	20	95	10

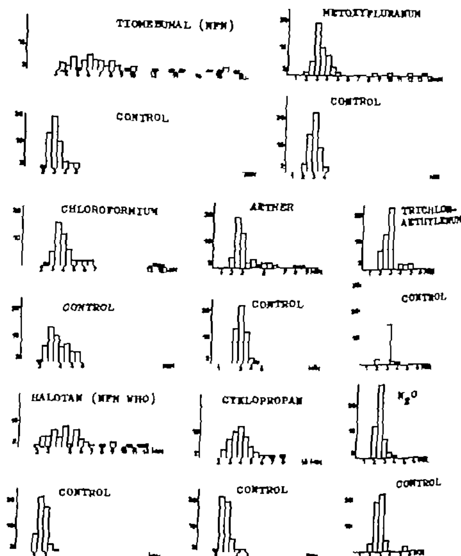


Fig. 2. Graphic representatio of experimental results.
Abscissa, survival time in minutes.
Ordinata, number of animals.

The mice anaesthetised with thiopentone had this injected intravenously 1 8 mg to each animal. The interval from the time of injection to the beginning of anoxia was 5 to 10 minutes.

Fig. 2 illustrates the distribution of mice according to survival within the anaesthetized and corresponding control groups. For certain anaesthetics a tendency is seen towards a shift of the survival time to the right, *i.e.* towards prolonged survival of the animals exposed to anoxia during anaesthesia.

Table 2

Average survival time (in minutes) of nonanaesthetised control mice and anaesthetised mice, and percentage prolongation of the survival time.
(In calculating the average survival times the highest values have been excluded)

Anaesthetic	Control mice (min.)	Anaesthetised mice (min.)	Prolongation of survival time (%)
Thiomebumal (NFN)	3.13	7.95	154.0
Aether ad narcosin (NFN)	3.06	3.06	0.0
Chloroformium ad narcosin (NFN)	3.57	3.98	11.5
Trichloraethylenum ad narcosin (NFN)	2.65	2.87	8.3
[illegible]	2.86	4.12	44.1
[illegible]	2.69	4.96	84.4
[illegible]	2.94	3.30	12.2
[illegible]	2.78	2.52	9.4

Leakage of the mouse bottle may be suspected for the longest survival times, especially when high values were found for both mice in the same bottle in the same experimental series. Excessively high values were ruled out.

Even on exclusion of these high values, a shift to the right, *i.e.* prolonged survival, was found for several anaesthetics.

Both the average prolongation of survival and the percentage prolongation are recorded in table 2.

Discussion

The results of ARNFRED & SECHER's (1962) investigations into the protective action of thiopentone against anoxia have been confirmed by us. We have had similar results with some other anaesthetics. Our survival times were a little shorter than those found by ARNFRED & SECHER, presumably because our experiments were carried out at 32–34 whereas theirs were performed at 24 the lower temperature may prolong survival. It is a well-known fact that administration of barbituric acid is accompanied by a fall in body temperature. However this fall can hardly alone explain the prolonged survival observed with anoxia during barbiturate anaesthesia.

Non-anaesthetised mice exposed to anoxia will develop severe convulsions, with a consequent excessive rise in metabolic rate, and will then die. As shown by BOLLMAN, FAZIO & FAULCONER (1951), raised metabolism reduces tolerance to anoxia. We therefore had reason to expect a shorter survival of non-anaesthetised than of anaesthetised mice, when both were exposed to anoxia. Thus, a moderately prolonged survival time is no evidence of a direct protective action of an anaesthetic against the harmful

effects of anoxia. The modest prolongation seen with animals anaesthetised with chloroform trichloroethylene or methoxyflurane amounting to 8% and 12%, may accordingly represent what is to be expected when convulsions are prevented during anoxia.

Absence of a similar prolonged survival by ether-anaesthetised animals is, perhaps, explainable by the long interval from the beginning anaesthetisation to the occurrence of sleep and the appreciable excitation with a consequent rise in metabolic rate.

A statistical analysis of the material gave a significant difference ($p < 0.01$) between the survival times for thiomebumal, halothane, cyclopropane and methoxyflurane. Finally a significant *shortening* of survival time was noticed for nitrogen monoxide. The prolongation observed for methoxyflurane was, however so small that we are inclined to regard it as merely due to this substance's prevention of the convulsions associated with anoxia.

An appreciable prolongation in survival time was only obtained with thiomebumal, cyclopropane and halothane. The animals given these anaesthetics survived for so long that other factors besides the prevention of convulsions must have played a part. These three drugs possessed a genuine protective action against anoxia. We cannot decide from the results presented here whether the mechanism of action is the same for all three drugs or how this protection is effected.

Summary

Several anaesthetics have been investigated for their effect on the tolerance of mice to anoxia.

True protective action was demonstrated for thiopentone (thiopental), halothane and cyclopropane.

Moderate prolongation of survival time was demonstrated for chloroform, trichlorethylene, and methoxyflurane, presumably due to anoxia not being associated with convulsions during anaesthesia.

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Acute Toxicity of Sodium Fluoride for Rhesus Monkeys and Other Laboratory Animals.

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During the past few years pharmaceutical preparations of sodium fluoride have been made available for the prevention of dental caries. Further fluoride is found in some foods. Thus studies of its pharmacology are of significance. While studying the biological effects of sodium fluoride in this laboratory (Lu *et al* 1961 Rice & Lu 1963) it was considered of interest to determine the acute toxicity including the lethal dose of sodium fluoride for Rhesus monkeys. The acute lethal dose of fluoride was also determined for squirrel monkeys and rats.

Methods

Six male Rhesus monkeys, weighing about 2 kg., were given sodium fluoride in aqueous solution by slow intravenous infusion. The experimental procedure was similar to that used by Leone *et al.* (1956) on dogs. The rate of infusion was 1 mg/kg/min. in terms of fluoride ion. Electroencephalogram (E.E.G.), electrocardiogram (E.C.G.), respiration and arterial blood pressure were recorded on a multichannel Grass electroencephalograph before and during the infusion of fluoride. A section of typical pre-treatment tracings is shown in fig. 1

For the recording of the EEG platinum electrodes were aseptically implanted. A few days before the infusion of fluoride, each monkey was anaesthetized with pentobarbital. A long sagittal incision was made on the scalp to expose the calvarium. Four holes were then drilled through the skull, two on each side and about 8 mm from the midline. The left and right frontal holes were located about 15 mm anterior to the sulcus centralis (coronal suture) and the two parietal holes about 15 mm posterior to this sulcus. The dura mater was slit under these holes. Platinum electrodes were then

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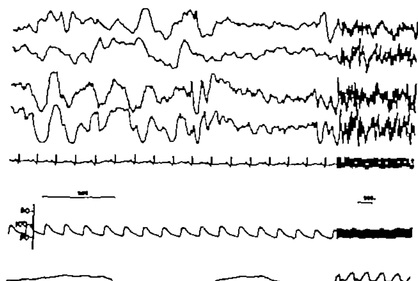


Fig. 1 Section of typical tracings btained by use of multi-channel Grass electroencephalograph in monkey before the infusion of sodium fluoride. The tracings from top to bottom are electrocorticograms 1 RF LF 2, RP–LP 3, RF–RP 4 LF–LP electrocardiogram (Lead II) blood pressure (systole up-stroke) base line for blood pressure tracing and respiration (inspiration up-stroke).

placed beneath the dura mater and secured in place with dental cement. After the operation, the monkeys were placed in restraining chairs. These chairs permit only limited movement. The monkeys were able to feed themselves when food was provided, but they were unable to reach the implanted electrodes on the head.

On the day of the infusion, with the monkey still in the chair a femoral artery and femoral vein were exposed and cannulated under local anesthesia. The venal cannula was then connected to a syringe for infusion of fluoride. The arterial cannula was used to record blood pressure through a Statham pressure transducer Respiration was registered by tying a skin suture on the chest to a Statham force-displacement transducer The platinum electrodes were connected to the electroencephalograph by means of insulated thin wires.

Throughout the infusion the monkeys were observed for size and reactivity of the pupil, emesis, defaecation, urination and other behavioral changes. Samples of blood were taken before the infusion began and immediately after the death of the monkey The levels of calcium and magnesium were then determined in the serum of these samples by the method of ROBINSON & RATHBUN (1959).

The infusion of fluoride was continued until respiration stopped. The dose that caused respiratory arrest was considered the lethal dose. This was determined on the six Rhesus monkeys as well as on six male squirrel monkeys and six male albino rats. The fluoride was throughout infused intravenously at the rate of 1 mg/kg/min.

Results

Lethal doses

The acute lethal doses of sodium fluoride are listed in table 1 The Rhesus monkeys and the rats were about equally sensitive to fluoride per

Table 1

Acute lethal dose of sodium in terms of fluoride ion, administered by intravenous infusion at 1 mg/kg/min.

Species	Lethal dose ± s.e.m. (mg/kg)
Rhesus monkeys	65.8 ± 6.32
Squirrel monkeys	26.6 ± 3.12
Albino rats	60.0 ± 2.40

unit of body-weight. The squirrel monkeys, on the other hand, were considerably more sensitive. These monkeys were found upon autopsy to be heavily infested with intestinal parasites. It is possible that the infestation increased the susceptibility of the monkeys by adversely affecting their general state of health.

Blood pressure and heart rate

The average femoral arterial blood pressure and heart rate of the Rhesus monkeys are listed in table 2. After an infusion of 20 mg/kg, there was a slight but insignificant decrease in the systolic and diastolic blood pressures.

Table 2

Effects of sodium fluoride on blood pressure, heart rate and respiration in Rhesus monkeys (mean ± s.e.m.).

Dose of NaF	Blood pressure Systolic/diastolic, in mm Hg	Heart rate/min.	Respiratory[3]) rate/min.
None (pre-treatment)	141 ± 7.6/101 ± 10.5	213 ± 10.2	33.3 ± 2.91
10 mg/kg	138 ± 10.4/100 ± 9.7	229 ± 5.8	38.3 ± 4.08
20 mg/kg	113 ± 12.9/81.6 ± 9.5	234 ± 9.2	39.6 ± 4.30
30 mg/kg	109[1]) ± 10.4/81.7 ± 5.0	185 ± 20.8	39.7 ± 3.73
40 mg/kg	100[2]) ± 7.2/73.1 ± 7.3	185 ± 15.7	43.7 ± 4.13
50 mg/kg	82[2]) ± 12.1/59[2] ± 6.1	137[1]) ± 24.4	32.0 ± 7.05

[1]) P 0.05
[2]) P 0.01
[3]) The figure is the mean for 4 monkeys; the rates of the other two monkeys became irregular

A significant fall in blood pressure was noted after about 50 % of the lethal dose had been given. The terminal systolic and diastolic pressures were, respectively 49.2 ± 9.38 and 35.8 ± 6.42 *mm Hg*. The readings obtained when the monkeys had received about 95 % of the lethal dose were considered terminal.

The heart rate fluctuated during the course of the infusion. An unequivocal decrease in heart rate was observed only after infusing 50 mg/kg. The terminal heart rate was 72.3 ± 7 37 per minute.

Respiration.

There was a slight but progressive increase in the rate of respiration (table 2) during the first 40 minutes of infusion. As the infusion continued, however the respiratory rate decreased. There were also dyspnoea, manifest mainly in the inspiratory phase, and occasional gasping (fig. 2).

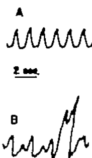


Fig. 2. Tracings of respiratory movement of a monkey that died after receiving 50 mg of fluoride per kg body weight. A. Control (pre-treatment) B. After infusing 45 mg/kg There were occasional gasping and inspiratory dyspnoea.

E.C.G

The most consistent change in the electrocardiogram was a progressive reduction in the amplitude of R waves. This occurred in all monkeys and usually became noticeable after about 80 / of the lethal dose of fluoride had been infused. Accentuation of T waves was observed in 5 monkeys. This was also a progressive change, and it occurred simultaneously with

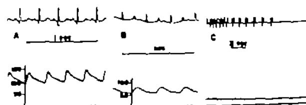


Fig. 3 Electrocardiogram (Lead II) and blood pressure tracing of monkey that died after receiving 74 mg fluoride per kg body weight. A. Control (pre-treatment). B. After receiving 65 mg/kg. There were bradycardia, prolongation f P–R interval, decrease in the amplitude of R waves and increase the amplitude of T waves. The blood pressure was much lower than the control value. C. Terminal events recorded at a slower speed. The T waves were markedly accentuated, and the S waves were also predominant. The heart rate progressively decreased, and finally the heart beat stopped. The electrocortical activity and respiration stopped about 30 seconds before the cardiac arrest in this monkey

the diminution of R waves. Elevated S–T junction was observed in 1 monkey along with the changes in the R and T waves. Bradycardia and prolonged P–R interval were increasingly noticeable after the monkeys had received 85 / of the lethal dose of fluoride. Auricular fibrillation was found in 1 monkey and partial auriculo-ventricular heart block in 2 monkeys shortly before death Fig. 3 shows some of the changes in E.C.G during the infusion of fluoride.

E.E.G

No appreciable change in the E.E.G was noted in these monkeys during the first 40 minutes of infusion. As more fluoride was infused, the fast waves (30–40 cyc./sec.) became less evident. At the same time the slow waves ($\frac{1}{2}$–1 cyc./sec.) were increasingly more predominant. The amplitude of the intermediate waves (5–10 cyc./sec.) remained relatively constant until about 90 / of the lethal dose had been infused Terminally the fast and intermediate waves disappeared from the E.E.G a few minutes before respiratory arrest, whereas the cessation of slow waves usually coincided with that of respiration. Sections of typical E.E.G tracing are shown in fig. 4

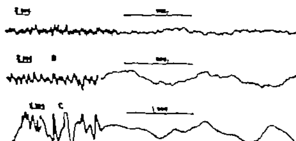


Fig. 4 Electroencephalogram (right front t left front) of a monkey that died after receiving 50 mg of fluoride per kg body weight. A. Control (pre-treatment). B. After infusing 40 mg/kg. The amplitude of the slow waves increased. C. After 49 mg/kg. The slow waves were more predominant, and the fast waves had disappeared.

Other manifestations and terminal events

There were no obvious signs of toxicity in any monkey during the first half of the infusion. Later the monkeys appeared depressed, and there were alternate periods of wakefulness and sleep Repeated defaecation was seen in all monkeys, and the stools were generally bloody No vomiting was noted. Muscle fasciculations were observed in two monkeys after infusing 40 mg/kg. One other monkey had 2 episodes of clonic

convulsions at 5 and 10 minutes before death. Pupils were normal in size and reactive to light until shortly before death, when they became dilated and fixed Frothing at the mouth was seen in all monkeys before death.

The heart beat, as evidenced by pulsations on the blood pressure tracing, and respiration stopped more or less simultaneously in 2 monkeys. Respiratory arrest preceded cardiac arrest by about one minute in the other 4 monkeys. In two of these monkeys, E.C.G s with regular rhythmicity were recorded for more than 5 minutes after "death when the pulse, respiration and pain and pupillary reflexes disappeared.

Blood calcium and magnesium.

The serum levels of calcium and magnesium before and after infusing lethal doses of sodium fluoride are summarized in table 3 There was marked reduction in the Ca level, but the Mg level was only slightly lowered

Table 3

Serum levels of Ca and Mg, expressed in mg/100 ml, in monkeys bef re and after a lethal l dose of sodium fluoride (mean ± s.e.m).

	Calcium	M gnesium
Before NaF	9 63 ± 0 266	1 68 ± 0.112
After NaF	3.55 ± 0.780	1 40 ± 0 123

Discussion

The intravenous lethal dose of sodium fluoride for dogs reported by LEONE *et al* (1956) were smaller than those found for the Rhesus monkey and the albino rat (table 1) This may be a true indication of a difference in sensitivity between the dog and the other two species. The slower rates of infusion in the dogs were unlikely to be the cause of their greater sensitivity since the lethal doses of fluoride were essentially identical when infused at 5 4 mg/min and at 1 1 mg/min. It may be noted that cardiac arrest followed respiratory arrest in four of the six monkeys. There was no evidence of ventricular fibrillation, which has been reported in the dog (LEONE *et al* 1956) and the cat (GOTTDENKER & ROTHBERGER 1935) It is possible that the cardiac susceptibility of the dog is responsible for the greater toxicity of fluoride to that species.

The early reactions to toxic doses of sodium fluoride in monkeys were

central nervous system depression and decrease in blood pressure. The latter has also been observed in some other species, e.g., the dog (LEONE *et al* 1956 GOTTDENKER & ROTHBERGER 1935), the cat (GOTTDENKER & ROTHBERGER 1935) and man (RABINOWICH 1945) The drop in blood pressure was accompanied by bradycardia and was probably due to a decrease in cardiac out-put resulting from the bradycardia and possibly also to peripheral vasodilatation. Vasodilatation has been shown to occur in the dog (BISHOP *et al* 1945) and in the isolated rabbit ear (HETENYI *et al.* 1954) Though the mechanism underlying the bradycardia is uncertain, it is reasonable to assume that, in view of the profound sedation induced by fluoride, the central cardiovascular centres would be depressed. Another factor that might be considered is cholinesterase inhibition by fluoride, which would increase concentrations of acetylcholine at the vagal terminals. Moreover sodium fluoride causes hypocalcemia, and it is well-known that this state will cause increased excitability of the autonomic nervous system.

Respiratory depression, which was observed in the monkeys and has also been observed in other mammalian species, could also be a consequence of central nervous system depression. DE NITO (1928) found that depression of respiration during fluoride intoxication in dogs could not be prevented by atropine which would suggest that respiratory depression is not produced through peripheral parasympathetic activity

During infusion of sodium fluoride in monkeys, the E.E.G changed from fast, low-amplitude activity to slow high-amplitude waves This is consistent with the observed depression of the central nervous system. It may be noted in passing that chronic feeding of small doses of sodium fluoride causes an increased susceptibility of the central nervous system to certain chemical and physical stimuli. This has recently been reported for rats (LU *et al* 1961) and has been observed in Rhesus monkeys (unpublished results) These monkeys had been given oral sodium fluoride to provide 2 mg of fluoride per kg body weight daily for eight weeks. At the end of this period lower thesholds to pentylenetetrazole seizures and longer duration of the seizures were observed in the fluoride-treated monkeys than in the untreated controls.

Defaecation with bloody stools was observed during acute intoxication in monkeys. This has also been observed by LEONE *et al* (1956) in dogs It is possible that this was due to the development of hemorrhagic lesions similar to those seen by SIEGFRIED (1901) in the stomach and intestines of rabbits after subcutaneous lethal injections of sodium fluoride.

Such lesions may cause increased peristalsis through irritation. This, along with increased parasympathetic activity caused by cholinestera- inhibition and by hypocalcemia, could account for the defaecation.

Summary

Slow intravenous injections of large doses of sodium fluoride into Rhesus monkeys caused a fall in blood pressure, bradycardia and depression of the central nervous system. The electrocardiogram showed a progressive reduction in amplitude of R waves and in some monkeys progressive accentuation of T waves. Auricular fibrillation and partial A–V block were less frequently noted. The electroencephalogram changed from fast low amplitude to slow high amplitude activity. Grossly depression and alternate periods of wakefulness and sleep were noted. Muscle fasciculations and clonic convulsions were occasionally seen. Repeated bloody defaecation occurred in all monkeys, but there was no vomiting. Frothing at the mouth was seen. Pupils were of normal size and reactive to light until shortly before death, when they became dilated and fixed. There was marked reduction in serum Ca, but the Mg level was only slightly lowered. Rhesus monkeys and rats were about equally sensitive to sodium fluoride, but squirrel monkeys were more sensitive than either

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The Effect of Codeine Tolerance on Hepatic Microsomal Drug Metabolism in the Rat.

By

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SANFILIPPO (1948) has postulated that the analgesic action of codeine may be due to its conversion to morphine by drug metabolic enzymes *in vivo*. JÓHANNESSON & WOODS (1964) found lower plasma concentrations of biotransformed morphine in codeine tolerant rats than in non-tolerant rats at 30 minutes after injection of codeine. It could therefore be postulated that the enzymic oxy-demethylation of codeine to morphine procedes at a slower rate in animals made tolerant to codeine than in non-tolerant rats. In order to investigate this hypothesis, the rate of O-dealkylation of codeine to morphine was studied in liver microsomes from rats made tolerant to the analgesic action of codeine. The specificity of the effect of codeine tolerance on the rate of hepatic drug metabolism was also studied. In the investigation reported here we have therefore, besides determining the conversion of codeine to morphine, studied the metabolism of eight other substrates in liver microsomes from codeine tolerant and non-tolerant rats.

Methods

For the experiments we used male Holtzman rats (180–300 g). They were kept under constant environmental conditions with free access to water and a commercial food preparation. Normal rats (control rats) are referred to here as non-tolerant

Codeine tolerance in the rats was produced by subcutaneous injections of increasing amounts in the morning and the evening of each day for approximately 4 weeks. The injection solution was 42 mg/ml of codeine phosphate in water Injections were given in the gluteal region. Each injection amounted to half the daily dose of the drug. The initial dose was about 15 mg/kg a day and the final dose was approximately 100 mg/kg (expressed as codeine base). The rats gained in weight during the tolerance period, but less than the controls.

This schedule is the same as that used by JÓHANNESSON & WOODS (1964). Their rats were highly tolerant to the analgesic action of codeine and morphine when tested 20 hours after the last dose in the tolerance period.

Preparation of tissue Twenty hours after the last dose in the tolerance period, all rats and controls were killed by a blow on the head. The livers were then immediately removed and homogenized on ice with a Potter homogenizer having a plastic pestle. Homogenates were prepared such that each gram of liver was suspended in 2 ml of cold isotonic (1 15 %) KCl to a total volume of approximately 3 ml. A supernatant fraction, containing microsomal and soluble enzymes, was prepared from the homogenates with a high speed angle centrifuge at 5° (9000 G).

In vitro determinations. One ml of the supernatant was incubated in a Dubnoff shaker-incubator at 37° with oxygen or nitrogen as the gaseous phase, for determining enzymic activities. Final concentrations of the cofactors were triphosphopyridine nucleotide (NADP) 1 1 $\times 10^{-4}$M glucose-6-phosphate 5 $\times 10^{-3}$M, nicotinamide 2 $\times 10^{-2}$M and $MgSO_4$ 5 $\times 10^{-3}$ M. The last cofactor was omitted from the benzpyrene pathway The concentrations of all cofactors were supraoptimal. Final volumes were brought to 5 ml and adjusted to pH 7 4 with 0.1 M phosphate buffer.

The pathways studied, the methods used in their assay and the substrate concentrations in μmoles per 5 ml incubation mixture were

1) the O-dealkylation of codeine to morphine (estimated by the colour reaction of SNELL & SNELL 1937) 10.0 μmoles
2) the side-chain oxidation of hexobarbital (enhexymal COOPER & BRODIE 1955), 3.0 μmoles
3) the ring sulphur oxidation of chlorpromazine (SALTZMAN & BRODIE 1956), 1.0 μmole
4) the reduction of the nitro-group of *p*-nitrobenzoic acid to yield *p*-aminobenzoic acid (FOUTS *et al* 1957), 12.0 μmoles
5) the reduction of the azo-linkage of neoprontosil to yield sulphanilamide (FOUTS *et al.* 1957), 7.5 μmoles
6) the hydroxylation of zoxazolamine to yield 6-hydroxyzoxazolamine (CONNEY *et al.* 1960) 3 0 μmoles
7) the hydroxylation of aniline to *p*-aminophenol (DIXON *et al.* 1963), 12.5 μmoles;
8) the hydroxylation of benzpyrene to yield hydroxybenzpyrene (CONNEY *et al.* 1957), 0.6 μmoles and
9) the N-dealkylation of aminopyrine to 4-aminoantipyrine (4-aminophenazone; BRODIE & AXELROD 1950), 40 0 μmoles.

The metabolite 4-aminoantipyrine was determined as described by BRODIE & AXELROD except that it was not extracted from the incubation mixture. Instead, the incubation mixture was treated with 15 ml of cold 6.67 % (w/v) trichloroacetic acid (total volume of incubate plus the trichloroacetic acid, 20 ml), and the protein was sedimented by centrifugation. Then, 4 ml portions of the "protein-free" supernatant were diazotized and coupled with α-naphthol as in the method of Brodie and Axelrod. This simplified procedure avoids the extraction step of the older method and was suggested by L. E. GAUDETTE (personal communication, 1964).

Disappearance of substrate was measured in following the metabolism of hexobarbital, chlorpromazine, zoxazolamine and benzpyrene. The appearance of the metabolites listed was determined in the other pathways studied.

The incubation time for the benzpyrene hydroxylation was 30 min., 60 min. for the nitro-group reduction, and 90 min. for the azo-group reduction. All other incuba-

tion times were 120 min. The results are given as nanomoles of the substrate metabolized, *or the product formed*, per incubation period and per gram liver (wet weight).

By the methods described, we tested whether brain tissue of non-tolerant rats could convert codeine to morphine. The results were negative.

Student's t test was used as a test of the "statistical hypothesis" (SNEDECOR 1956). The level of significance used for all determinations was P = or < 0.05.

Results

The results are given in table 1 They show that the hepatic microsomal drug metabolizing enzyme activities are lower in codeine tolerant than in non-tolerant rats. This difference is statistically significant for 8 out of 9 metabolic pathways studied, including 3 different hydroxylation path ways, 2 oxidation pathways and the oxy-demethylation of codeine. The enzymic N-demethylation of aminopyrine appeared to be decreased the decrease was, however not statistically significant for the number of animals involved.

Table 1

The effect of chronic administration of codeine on hepatic microsomal drug metabolism in the rat. The results are expressed as nanomoles of substrate metabolized, or product formed, per g wet weight liver and per incubation period. Each value represents the mean of determinations of enzyme activity for 6 rats ± its standard deviation.

Metabolic pathways (substrates → major metabolites)	Non-tolerant rats (controls)	Codeine tolerant rats
Codeine → Morphine	1800 ± 170	830 ± 190[2]
Hexobarbital → Ketohexobarbital	3010 ± 980	1020 ± 580[2]
Aniline → 4-Aminophenol	1060 ± 180	710 ± 140[2]
Aminopyrine → 4-Aminoantipyrine	690 ± 160	540 ± 110[3]
Benzpyrene → Hydroxybenzpyrene	230 ± 130	60 ± 50[2]
Zoxazolamine → 6-Hydroxy Zoxazolamine	910 ± 300	640 ± 240[2]
Chlorpromazine → Sulphoxychlorpromazine	380 ± 110	< 50 ± 0[2]
p-Nitrobenzoic acid → *p*-Aminobenzoic acid	1570 ± 260	1230 ± 280[2]
Neoprontosil → Sulphanilamide	2830 ± 450	1740 ± 670[2]

1) cf. [illegible] (1959)
2) values significantly lower than the corresponding control values
3) not significantly lower than the corresponding control value.

Discussion

The results indicate that chronic pretreatment with codeine depresses drug metabolizing enzyme activities located in the liver microsomes of the rat. The observed decrease in microsomal enzyme activities is apparently non-specific in nature, in so far as all the metabolic pathways studied were involved (table 1). It therefore seems unlikely that the reduced conversion of codeine to morphine in the codeine tolerant animals is the cause of the tolerance it is rather a result of tolerance development.

BOUSQUET RUPE & MIYA (1964) have found that chronic pretreatment with morphine decreases the metabolism of hexobarbital, meprobamate and morphine. Further experiments might therefore substantiate whether chronic administration of narcotic analgesics in general results in a physiological or pathological condition in which the rate of drug metabolism by the hepatic microsomal enzymes is decreased.

Microsomal drug metabolism is usually decreased when hepatic glycogen is reduced (FOUTS 1963) In this connection it should be recalled that morphine is a potent inhibitor of glycogen synthesis in the rat (KUN & ABOOD 1949) Although tolerance was developed to this effect of morphine on repeated administration, ABOOD KUN & GEILING (1950) found that the brain and liver from morphine tolerant rats still possessed significantly lower energy capacities than did brain and liver of control animals. *A priori* it may also be considered likely that the same conditions prevail after chronic treatment with codeine, caused either by the codeine itself or of the morphine biotransformed from it. It is indeed at the present time, purely conjectural whether this has any causal connection with the results presented here, but it seems to be a possibility worth mentioning.

Summary

Hepatic microsomal drug metabolizing enzyme activities are lower in rats made tolerant to the analgesic action of codeine than in non-tolerant rats. This inhibition is probably non-specific in nature. The reduced conversion of codeine to morphine in codeine tolerant rats is a resultant of tolerance to codeine rather than its cause

Acknowledgements.

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Determination of Bretylium in Biological Fluids.

By

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(Received August 21 1964)

Bretylium tosylate = [(*o*-bromobenzyl)-ethyl dimethylammonium toluene-p-sulphonate] a quaternary salt described by Boura, Copp & Green (1959) causes post ganglionic sympathetic blockade (Boura *et al* 1959) it is used clinically as a hypotensive agent. Various methods for its determination have been reported in the literature.

Duncombe & McCoubrey (1960) used two methods first, ion-exchange and determination as a complex with methyl orange, and secondly complex formation with cobalt thiocyanate (Ashbrook 1959). However these methods are tedious and did not appear very satisfactory to us, owing to high blank values.

Quaternary nitrogen compounds form with dipicrylamine complexes that are extractable into organic solvents according to Schill (1959) and Schill & Danielsson (1959), who used this reaction to determine neostigmine, propantheline and hexamethonium, the complex being extracted into chloroform. We have used their method as the basis of a rapid technique for determining bretylium in biological fluids.

Methods

Reagents and apparatus.

1 Dipicrylamine (Merck Darmstadt) 0.01 in benzene A.R.
2. Sodium hydroxide N and 0.1 N

All optical density measurements were made with a Unicam SP 1400

Schill (loc cit) reported that dipicrylamine (hexanitrodiphenylamine) was contaminated with pentanitrodiphenylamine and recommended purification by chromatography on Kieselguhr However thin layer chromatography on microscope slides coated with silica gel G (Merck) in a variety of solvents showed only one spot the dipicrylamine was therefore used without further purification.

Preparation of standard curve

Measured portions of an aqueous solution of bretylium were pipetted into 40 ml stoppered centrifuge tubes and diluted to a final volume of 5 ml. A drop of 0.01% phenolphthalein was added and then 0.1 N NaOH dropwise until the solution was just pink. Dipicrylamine solution in benzene 10 ml of the 0.01 % was added, and the tubes were shaken for 2 minutes and lightly centrifuged to clarify the benzene layer The optical density of this layer was measured at 420 mμ.

Typical results are given in table I.

Table 1

Optical density of benzene-soluble bretylium-dipicrylamine complex.

Bretylium µg added	O D (420 mµ)
20	0.113
40	0.249
60	0.366
80	0.499
100	0.621

Determination in urine

Bretylium tosylate, 11.8 mg, was dissolved in 10 ml of rat urine this solution was diluted with water to give suitable working concentrations. Measured portions were then analysed, as in the preparation of a standard curve, except that 5 N NaOH was used to adjust the pH

Typical results are given in table 2.

Table 2

Optical density of benzene-soluble bretylium-dipicrylamine complex.

Bretylium µg added	O.D (420 mµ)
23.4	0.137
46.8	0.292
70.2	0.423
93.6	0.575
117.0	0.720

Mean recovery bout 99 %

Determination in blood

Measured portions of a standard solution of bretylium tosylate were mixed with 0.5 ml of horse blood (Burroughs Wellcome) and diluted to

5 ml. Each solution was made just alkaline with 10 N NaOH and then assayed as in the preparation of a standard curve. The mean recovery over a concentration range 20–100 μg bretylium tosylate was 93 %.

Specificity

The drugs that did not form a complex with dipicrylamine under our conditions were amiphenazole, DL-amphetamine, histamine, meprobamate. Hydralazine and acetylcholine formed complexes insoluble in benzene. Guanethidine formed a yellow complex soluble in benzene

Conclusions and Summary

The reaction of dipicrylamine and quaternary nitrogen compounds yielding coloured complexes soluble in organic solvents provides a sensitive and rapid method for determining quaternary nitrogen compounds in biological fluids The method at present is sensitive to 10 μg/ml of bretylium the sensitivity could probably be increased at least four-fold by slight modifications. Extremely low blanks were obtained with blood and urine.

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Chemical Determination of Histamine in Tissues and Mast Cells.

By

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(Received September 1 1964)

Several chemical and biological methods have been described for the quantitative determination of histamine in samples from blood and tissues.

By using the rectal caecae of fowls BARSOUM & GADDUM (1935) developed a sensitive method for determining the histamine content of blood. Others have used an isolated preparation of the guinea pig ileum or the effect of histamine on the blood pressure of the cat (CODE 1937 RILEY & WEST 1953).

For determining the histamine content of blood plasma and cells LOWRY et al (1954) described a colorimetric measuring method that determines histamine in quantities as small as 0.01 μg.

CLOUET et al (1957) use a colorimetric determination that measures histamine in the range 0.5–20 μg. A fluorometric determination has been described by SHORE et al (1959) This method permits measurement of histamine in concentrations as low as 0.005 μg/ml.

Our study aimed at simplifying Lowry's method and investigating the applicability of the modification to determining histamine in tissues and in a suspension of peritoneal mast cells from the rat. Also the specificity of the method was studied in tissue specimens.

Methods

Deproteinization and extraction.

The removed tissue specimens, ranging in weight from 0.5–7 g, were placed whole in 25 ml centrifuge tubes and covered with 10 ml 15 % (w/v) trichloroacetic acid, and the tubes were closed with aluminium foil. The specimens were left for 18 hours at 70° and shaken until the tissue disintegrated. Histamine was then present in the fluid phase. Renewed treatment with trichloroacetic acid did not increase the histamine yield The remaining tissue was removed from the fluid by centrifugation or filtration through a small cotton-wool filter

Adsorption and elution

The histamine containing fluid was poured onto a Decalso column, consisting of a 3 cm layer of "Decalso F" ® which has a pore size between 60 and 90 mesh. The inner diameter of the column was 5 mm. Above it there is a reservoir holding 20 ml. Maximum adsorption of histamine (95–97 %) was obtained at pH 3–5 and at a flow rate not exceeding 1 ml/min. The adsorbed histamine was eluted with a solution of potassium bromide. The Decalso material has proved to vary somewhat in quality, so that the flow rate might exceed 1 ml/min. Therefore, standard specimens were run every day parallel with the tissue specimens.

Colour development and spectrophotometric determination.

The histamine was coupled to 2,4-dinitrofluorobenzene. This forms the yellow monodinitrophenyl derivative of histamine. According to LOWRY *et al.* (1954) the reaction is most complete, when the coupling is performed at pH 10 and at 60°. The coupling product may be measured spectrophotometrically and has an absorption maximum at λ 360 mμ.

Standard curve and sensitivity of the method

To determine the standard curve, 5 different concentrations of histamine hydrochloride (Ph Dan. 1948) were used, dissolved in a mixture of 2 parts modified Tyrode solution and one part 15 / (w/v) trichloroacetic acid. The modified Tyrode solution was a buffer solution (pH 7.3) containing 1 44 × 10^{-1} M NaCl, 3 × 10^{-3} M-KCl, 8 × 10^{-4} M-$CaCl_2$ 2H_2O 8 × 10^{-3} M Na_2HPO_4, 2H_2O and 3 × 10^{-3} M-KH_2PO_4 Each concentration was the subject of 5 single determinations. As blanks 2 specimens were analysed without addition of histamine. The concentrations used were 36 20, 18 10, 9 05 4,53, 2.26 and 0 μg/15 ml of histamine base. After 1.5 ml 4 N sodium acetate 3 H_2O had been added to the solutions, they were poured onto Decalso columns. The analysis was then continued as described under tissue analysis. The extinctions found are given in table 1 The blank value was between 0.020 and 0.030. It will be seen that the histamine concentrations and the extinctions are linearly related within the range studied. The sensitivity defined as the amount of histamine (μg base)

Table 1

Correlatio between extinctio and histamine concentration, each based on 5 separate determinations. Two blanks were included.

Added histamine (μg base) per 15 ml	Extinction Mean ± s.e.m.	3 × s e.m.	Sensitivity μg histamine base
0	0.021		
2.26	0.082 ± 0 006	0.018	0.67
4.53	0.144 ± 0.005	0.015	0.54
9 05	0.258 ± 0.002	0.006	0 22
18.10	0.501 ± 0.007	0.021	0.80
36.20	1 010 ± 0 006	0.018	0.67
		Mean	0.58

corresponding to an extinction of 3 × s.e.m., was calculated as 0.58 µg histamine base. Thus, the method permits determination of histamine in amounts greater than 0.6 µg histamine base.

Analytical technique

The histamine content is given as µg histamine base/g tissue. In a suspension of peritoneal mast cells from the rat the histamine content was calculated as µg histamine base/ml.

For determination of histamine in tissues adult albino rats (150–200 g) were used. The blood was collected by bleeding from the carotid arteries. The total brain was removed, leaving the pituitary gland *in situ*. Total lungs and liver were used and about ½ g small intestine (jejunum plus ileum with attached mesentery). The intestine was carefully cleaned of intestinal contents. After depilation, a piece of skin (cutis plus subcutis) was removed from the anterior part of the back, the skin being separated with a scalpel from the underlying fascia and musculature. With a hollow punch a circular piece of skin, about 1 g, was punched out. All tissues were weighed fresh and stored for analysis at −16

The tissues were treated with trichloroacetic acid as described under *Deproteinization and extraction*. However, 80.0 ml 15 % (w/v) trichloroacetic acid were used for the liver. After careful shaking or stirring, the tissue will fall apart and is then removed from the histamine-containing fluid by centrifugation or filtration through a small cotton-wool filter. From the liver decantate 10.0 ml were removed and, after addition of 3.0 ml 4 N sodium acetate, 3 H_2O adjusted to pH 4. Of the other tissues the total decantate or the filtrate was used, the same amount of sodium acetate being added as for liver. The solutions were poured onto a Decalso column, which was then washed with a few ml of water. The adsorbed histamine was eluted with 5.0 ml 40 % (w/v) potassium bromide solution poured onto the column almost at boiling point (about 80–90°). The eluate was collected into 25 ml centrifuge tubes containing 0.40 ml sodium diethyldithiocarbamate 0.0125 % (w/v). To this were added 2.5 ml carbonate buffer (pH 10) containing 2×10^{-1} M-Na_2CO_3 and 10^{-1} M $NaHCO_3$. To this mixture was added 0.20 ml 2,4-dinitrofluorobenzene 2 (w/v) in absolute ethanol, and the whole was shaken for one minute. The specimens were kept on a water bath at 60° for 30 min., and 4.00 ml methyl-n-hexyl-ketone were added. The mixture was shaken for half a minute and centrifuged for a few minutes. The coupled histamine was now present in the supernatant ketone layer. Of this phase 3.00 ml were transferred to a centrifuge tube together with 3.00 ml 2 N-HCl. The mixture was carefully shaken for 3 min. and centrifuged for a few minutes. The histamine derivative was now present in the acid phase. The supernatant ketone layer was removed, and the extinction was measured spectrophotometrically in the acid phase by means of a Beckman DU spectrophotometer.

From the standard curve the amount of histamine poured onto the column and corresponding to the extinction may be read. This amount corresponds to the histamine content of the removed tissue, except for the liver, from which a measured portion was removed for analysis.

For analysis of the histamine content in a suspension of mast cells from the rat peritoneum the above mentioned rats were used. Of a modified Tyrode solution, heated to 38° 18 ml were injected intraperitoneally. The rat was killed 30 sec. later, the

abdomen cut open and the peritoneal fluid evacuated. To 10.0 ml of this suspension of peritoneal mast cells 5.0 ml 15 % (w/v) trichloroacetic acid were added to liberate the histamine bound intracellularly. After standing for 20 min. the suspension was centrifuged (4000 r.p.m. for 20 min.). To the decantate were added 1.5 ml 4 N sodium acetate, 3 H_2O and the mixture was poured on the Decalso column. The analysis was then continued as described under tissue analysis.

Technique for assessing the specificity of the method. To investigate whether the spectrophotometric activity measured in the tissue and mast-cell specimens corresponded with the histamine, the specimens were subjected to enzymatic treatment with diamine oxidase (histaminase) and a paper chromatographic study.

As already mentioned, the tissue was treated with 10.0 ml 15 % (w/v) trichloroacetic acid. In order to remove the trichloroacetic acid from the histamine containing fluid, the fluid was shaken 3 times with 60 ml ether (for Analysis). The aqueous phase was placed on a water bath at 60° for one hour. The volume then was about 5 ml and the pH of the fluid about 4. Two portions of 2.00 ml were removed. To each were added 2 ml water and 0.32 ml buffer solution (pH 6.5) containing 1.17 M-KH_2PO_4 and 0.59 M Na_2HPO_4, 2 H_2O. To one portion were added 4.00 ml 4.00 mg/ml diamine oxidase solution (diamine oxidase isolated from hog kidney. Sigma Chemical Company U.S.A.) and to the other a corresponding volume of water; both solutions were then left at 37° for 12 hours. To the samples were added 4.0 ml 15 % (w/v) trichloroacetic acid and 1.4 ml 4 N sodium acetate, 3 H_2O and the precipitated protein was centrifuged off. The decantate was poured onto a Decalso column and the analysis continued as described under tissue analysis. With the diamine oxidase solution used the spectrophotometric activity of a standard sample of 30 μg histamine base could be completely destroyed.

In a paper chromatographic study of the tissue extract the tissue was treated as mentioned under analytic technique. Of the acid phase, which consists of coupled histamine dissolved in 2 N-HCl, 0.05 ml was applied as a line of spots on Whatman paper No. 1, the paper being dried during the application in a current of hot air. An ascending chromatogram was developed in a solvent consisting of 80 parts n-butanol (for Analysis) and 20 parts 2 N HCl. The developed zones were eluted with 3.00 ml 2 N-HCl and the extinction measured spectrophotometrically in the acid phase. Standard tests showed a single zone with an R value at 0.55 (fig. 1). On elution of the zones, about 90 % of the amount of histamine applied to the paper was recovered in the eluate.

Results

Recovery

The recovery was studied for the tissues named and in a suspension of peritoneal mast cells. The tissues were removed as described under analytical technique and suspended in water by a Braun Multimix homogenizer. The amount of tissue was such that the aqueous homogenate represented a tissue content of about 0.1–0.2 g/ml, but for skin only 0.05 g/ml. From the homogenate, portions of 5.00 ml were removed; histamine was added in increasing quantity, each dosage receiving two separate determinations (table 2). For determining the content of tissue

histamine in the homogenate, 4 samples of homogenate to which no histamine was added were run for each organ. Further 4 standards were prepared by dissolving 66.2 µg histamine hydrochloride (corresponding to 40 µg base) in 5.00 ml water. The samples were shaken, and 5.0 ml 30% (w/v) trichloroacetic acid were added. Thus the same concentration of trichloroacetic acid was used in the recovery experiments as in the determinations of histamine in the tissues. After the specimens had been left for 18 hours at 70° the tissue was centrifuged off the histamine containing fluid. To the decantate 3.0 ml 4 N sodium acetate, 3 H_2O were added, and the mixture was poured onto a Decalso column. The analysis was then continued as described under analytical technique. The recovery was calculated as the ratio between the extinction found in the homogenate sample with histamine added and the sum of the extinctions measured in the standard and homogenate samples without addition of histamine. The percentage recovery will be seen from table 2.

Table 2

Recovery of histamine base added to homogenates of tissues or suspension of peritoneal mast cells.

Added µg histamine base per 5.00 ml homogenate	Recovery (percentage)					
	Skin	Lung	Liver	Brain	Intestine	Mast cells
40.0	91	87	97	99	86	91
40.0	100	92	94	100	86	89
20.0	113	89	96	97	93	93
20.0	92	91	94	105	97	93
10.0		91	102	93		92
10.0	95	92	96	95	99	97
5.0	99	93	104	92	93	86
5.0	108	103	91	104		88
2.5		103	95	97	94	94
2.5		105	97	101	85	89
Mean s.e.m.	100 3.1	95 ± 2.1	97 ± 1.2	98 ± 1.4	92 ± 1.9	91 ± 1.0

For the recovery in a suspension of peritoneal mast cells, the mast cell suspension mentioned under analytical technique was used. From this suspension samples of 5.00 ml were removed and treated like the tissue homogenates. The recovery is recorded in table 2.

Specificity

LOWRY *et al* (1954) studied the specificity of the method for a number of amines. They found that histidine, creatinine, glutamine and trypta

mine do not interfere with the histamine determination, whereas spermine, spermidine, putrescine, cadaverine, histidinol and agmatine do. In our investigation the following biogenic amines studied were 5-hydroxytryptamine (serotonin) creatinine sulphate, 5-hydroxytryptamine hydrogen maleinate, tyramine HCl, dopamine HCl, noradrenaline bitartrate and acetylcholine. The analysis was performed with 100 µg of the substances named, both with and without heating the trichloroacetic acid solution at 70° for 18 hours. None of the amines gave extractions greater than the blank values.

Histamine content of tissues

The histamine contents of the rat tissues studied are shown in table 3.

Table 3

Histamine activity" of tissue extracts of suspension of peritoneal mast cells from the rat. The content of histamine is given in µg "histamine base"/g tissue or µg "histamine base"/ml suspension, respectively.

Mean ± s.e.m. calculated.

Brain	Lung	Liver	Skin	Intestine	Blood	Mast cells
10.0	6.7	12.2	23.4	26.1	<0.6	0.8
8.6	6.5	13.8	36.1	35.9	<0.6	0.6
8.9	6.3	11.7	41.4	24.5	<0.6	1.8
8.3	7.3	14.4	25.8	31.9	<0.6	1.1
8.3	6.5	12.5	29.4	24.5	<0.6	1.9
8.0	5.3	14.0	22.6	20.3		1.8
8.7	4.3		25.4	18.5		0.8
8.7	8.0		24.2	22.8		0.7
8.9	6.0		26.1	18.3		
7.8	9.9		26.1	15.0		
8.6 ± 0.2	6.7 ± 0.5	13.1 ± 0.5	28.1 ± 1.9	23.8 ± 2.1	<0.6	1.2 ± 0.2

The values found for brain and liver are at marked variance with previous authors' findings by biological and spectrofluorometric measurement (table 4). For further investigation of the spectrophotometric activity of extracts from these two organs, the tissues were subjected to paper chromatographic analysis as well as to the enzymatic treatment.

The liver extract chromatogram consisted of 4 yellow zones. None of them could be identified as due to histamine, because each of their R_F values differed from that of the standard sample (fig. 1). In an area

Table 4

Rat tissue levels of histamine reported in the literature as µg histamine base/g tissue and µg/ml blood or suspension of peritoneal mast cells

Tissue	Methods for determination of histamine			Authors
	biological	colorimetric	fluorometric	
Brain	0.2–0.3			Moran & Westerholm (1963)
		4.3		Clouet *et al.* (1957)
			0.2–0.4	Shore *et al.* (1959)
			ca. 0.2	Zachariae (1963) pers. comm.
Lung	9			Parratt & West (1957)
	8			Telford & West (1961)
			1.7–6.8	Shore *et al.* (1959)
Liver	1			Parratt & West (1957)
	2			Telford & West (1961)
	0.3			Riley & West (1953)
			1.1–2.0	Shore *et al.* (1959)
Skin	23			Parrat & West (1957)
	31			Telford & West (1961)
	38			Hvidberg *et al.* (1961)
			25	Zachariae (1964)
Intestine	24			Telford & West (1961)
	18			Riley & West (1953)
Blood	0.1			Code *et al.* (1961)
Mast cells		4.3		Van Arsdel & Bray (1961)
			1.5	Zachariae (1963) pers. comm.

corresponding to the histamine zone in the standard specimen there was a trace of a zone showing a spectrophotometric activity about 18 % of the total (table 5).

Treatment of the liver extract with diamine oxidase showed a reduction in colorimetric activity of about 12 % (table 5).

These experiments show that only about 15 % of the measured activity can be ascribed to histamine. Since the activity apart from that of interfering substances, corresponds to 13.1 µg histamine base (Table 3), the real histamine content must be 15 % of 13.1 = 2.0 µg histamine base per g liver tissue (table 5).

The chromatogram of brain extract is shown in fig. 1 No activity was demonstrated in the area corresponding to the zone in the standard specimen.

Table 5

Corrected histamine contents of tissues and of suspension of peritoneal mast cells from the rat.

Tissue	Spectrophotometric activity: % found at R_F 0.55	Spectrophotometric activity: % decomposed with diamine oxidase	Histamine in % of the total spectrophotometric activity	Real histamine content in tissue (μg base/g or ml)
Brain	0; 0 0	0 0 0	0	<0.6
Lung	23 19	28 20 17	22	1.5
Liver	18	24 0 13	15	2.0
Skin	71 81	67	72	20
Intestine	27	22 43 30	30	7.1
Mast cells	100	100	100	12

The activity from the brain extract did not alter on treatment with diamine oxidase

The findings (table 5) are in exact accordance with the low histamine content reported in the literature, a content below the amount measurable by the method here under consideration.

The other tissues were studied in the same way and the chromatograms are shown in fig. 1 The part of the measured activity that can be identified as histamine, on the basis of the activity found on the chromatogram in the zone with R_F 0 5 and of the decrease in tissue activity caused by the enzymatic treatment, will be seen from table 5

The study shows that there will be to varying extents substances interfering with histamine determination in the lungs, skin and intestine, but not in a suspension of peritoneal mast cells.

Discussion

The method described above has given values for the histamine contents of rat brain and liver (table 3) at marked variance with values reported in the literature (table 4) Chromatographic and enzymatic studies have shown that the discrepancy is due to interfering substances that are not sensitive to diamine oxidase (histaminase) but are included in the spectrophotometric analysis The liver findings have therefore been corrected by chromatographic determination and enzymatic breakdown of the histamine. The corrected value (table 5) is in conformity with the findings in the literature.

In the brain practically the entire activity measured is derived from the interfering substances. Accordingly the values for histamine contents of rat's brain after treatment with morphine and of control rats brain

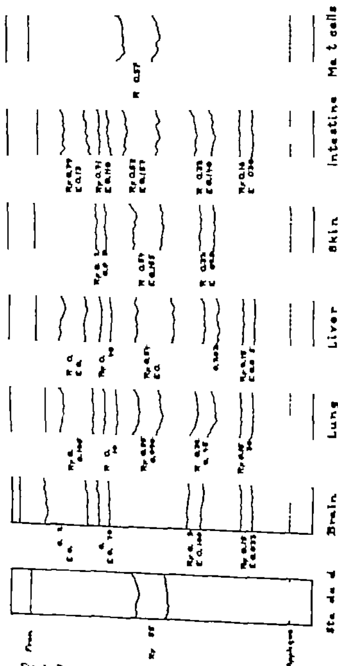


Fig 1 Paper hromatograms of tissues and of suspension of peritoneal mast cells from the rat.

reported in a previous communication (JÓHANNESSON & NORN 1963) must be withdrawn. It is impossible to correct the measured spectrophotometric activity in the brain, as the sensitivity of the method is too slight.

Measurements on the rat lung, skin and intestine show that these tissues contain various amounts of substances that interfere with the analysis. The corrected values correspond to the findings of previous authors (table 4).

On the other hand, chromatographic as well as enzymatic studies show that the method is specific for the measurement of histamine in a suspension of peritoneal mast cells. The method should therefore be restricted to determining histamine in these cells.

The method described has been used to investigate the influence of antirheumatics on the release of histamine after an antigen-antibody reaction in a suspension of peritoneal mast cells from sensitized rats. If the rats are treated towards the end of the sensitization period with antirheumatics *in vitro* treatment of the removed suspension of mast cells with antigen (horse serum) will liberate a smaller percentage of the total histamine content than in the control group not so treated. Only clinically potent antirheumatics show a significant inhibition of histamine liberation. This correlation is not obtained when antirheumatics are added direct to the mast cell suspension before the addition of horse serum. An account of this study is being prepared for publication.

Summary

A chemical method for determining the histamine in various tissues and in a suspension of peritoneal mast cells from the rat permits amounts of histamine base as low as 0 6 µg to be determined. The recovery in the tissues studied is between 90 and 100 %. All the tissues studied contained substances interfering with the determination of histamine. In the brain practically the entire activity measured is due to these substances the sensitivity of the method does not permit a correction for them. For the other tissues, it is possible to correct the values so corrected the values are in keeping with those reported in the literature. On the other hand, the method is specific and well-suited for determining histamine in a suspension of mast cells, but the histamine content of the blood is too low to be determined by the method.

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Acta pharmacol et toxicol. 1965 22, 126–130

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Interfering Substances by Determination of Poisons in Autopsy Material. IV Tryptamine.

By

Bent Kempe

(Received September 4 1964)

In the work reported here we studied tryptamine, one of the impurities observed by spectrophotometry in forensic chemical analyses of organ material for presence of ordinarily occurring alkaloids. A description is given below of how to isolate and identify the substance and of methods for separating it from commonly occurring alkaloids appearing in the same extraction phase.

Technique

Physical constants were determined as described previously (KEMPE 1964).

A single human liver stored in the minced state for about 3 years at 4–5° was used as material for study The original forensic chemical examination in this Department had revealed no poisons of any kind in the liver It was putrified.

Extract of the liver *was prepared* as follows After adding 8 N-H_2SO_4 to acid reaction, 50 g liver tissue and 25 ml of 96 % ethanol were homogenised The homogenate was extracted three times, each with an equal volume of ether which was thrown away After adding 8 N-NaOH to strong alkaline reaction, it was extracted five times, each with an equal volume of ether The combined ether phases were dried with anhydrous sodium sulphate, filtered and evaporated to dryness on a steam bath. The residue was heated on a boiling water bath for about 1 minute with 20 ml of 0.2 N-H_2SO_4. The sulphuric acid solution was cooled under the tap to about 20° and filtered. The filtrate was rendered alkaline with 2 N NaOH and extracted three times, each with an equal volume of ether The combined ether extracts were dried with anhydrous sodium sulphate, filtered and evaporated to dryness on a steam bath. The residue was sublimed at about 90° at a pressure of 5 mm Hg. The sublimate, weighing about 1 mg, consisted of long glistening white crystals.

Results

The corrected melting point of the substance isolated by the abovementioned extraction was 103 Its infrared and ultraviolet spectra are shown in figs. 1 and 2.

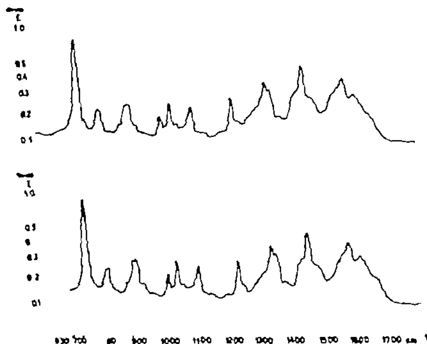


Fig 1 Infrared spectra of the substance isolated (upper curve) and tryptamine (lower curve).

The constants found are in fair agreement with the data given in the literature for tryptamine base, except for the melting point, which has been reported in the literature as 145–146° (EWINS 1911) and 118 (MANSKE 1932) To obtain material for comparison, we isolated the base from a purchased sample of tryptamine hydrochloride puriss. Fluka. This sample showed a corrected melting point of 252 calculation on the basis of a titration determination of the chloride content gave an equivalent weight of 200.6.

We used 20 mg of the purchased tryptamine, HCl, as starting material for isolation of the base. A few drops of N-NaOH were added to the chloride. After extracting five times, each with 5 ml of ether the combined ether extracts were dried with anhydrous sodium sulphate, filtered and evaporated to dryness on a steam bath. The residue was sublimed at 90° at a pressure of 5 mm Hg. The sublimate, weighing about 5 mg, consisted of long glistening white crystals.

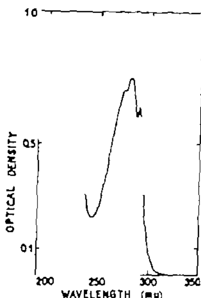


Fig. 2. Ultraviolet spectra of the substance isolated, in methanol.

The corrected melting point of the isolated tryptamine base was 103° which remained the same after cooling and renewed melting. Its infrared and ultraviolet spectra were noted. The corrected mixed melting point of the substance extracted from putrefied liver tissue with the isolated tryptamine base was 103

Comparison of the values obtained for the crystalline substance extracted from putrified liver tissue and the isolated tryptamine base showed these substances to be identical.

By chromatography on an activated silica gel-G plate, developed with PARTRIDGE & WESTALL (1948) mixture, purchased tryptamine, HCl, and the isolated and sublimed tryptamine base gave a yellow spot (R_F = 0.5) in response to spraying with diazotised p-nitroaniline (WICKSTRØM & SALVESEN 1952). The spot became brown on spraying with a base. The sublimed tryptamine base was dissolved in 30 ml of ether Gaseous HCl was passed into the solution, after which the ether was distilled off. The residue was dissolved in 1 ml of absolute alcohol. Ether was added to the solution, which was then left standing. The melting point of the precipitated crystals was 248 (corr). A recrystallized sample of the purchased tryptamine, HCl, had melting point 248° (corr), and this remained unchanged on mixing with the HCl salt obtained from the sublimate.

As seen in fig. 2, within the range of 200–350 mμ tryptamine showed maximum absorption at 279 mμ. The extinction of a 1 cm layer of a solution of 15 μg tryptamine per ml 0 1 N NaOH or 0.1 N HCl at 279 mμ being about 0.4 its presence will interfere with spectrophotometric

analyses for commonly occurring alkaloids. Separation of the substances has therefore been attempted.

Separation of tryptamine from commonly occurring alkaloids

The partition coefficient of tryptamine is 0.25 in 0.2 N NaOH/ether and 0.11 in 0.2 N NaOH/chloroform.

Chromatography on Whatman paper no. 1 prepared with a 0.1 M phosphate buffer of pH 6.3 and developed as described by BROSSI *et al.* (1955), *system A*, gave for tryptamine an R_F value of 0.07. In the paper chromatogram tryptamine was seen as a dark spot in light of 254 mμ. Spraying with the alkaloid reagents generally used in this department–iodoplatinate, modified Dragendorff and Fröhde – gave no colour reaction at the tryptamine site.

Our results seem to justify the conclusion that tryptamine is extracted in the same way as most commonly occurring alkaloids, but is separable from these by the paper chromatography procedure of BROSSI *et al.* *system A*. This is because most of the commonly occurring alkaloids have a higher R_F value than tryptamine. An exception is morphine (R_F 0.06), which, unlike tryptamine, is not extractable by chloroform or ether at strong alkaline reaction. As tryptamine does not react with iodoplatinate or Dragendorff's reagent, it will give no false reaction for alkaloids when these reagents are used for developing paper chromatograms.

Discussion

The difference between the melting-points found by us for tryptamine base and the several different melting-points quoted in the literature may be due to polymorphism, as the latter were determined after recrystallisation. The base isolated by us was sublimed under reduced pressure (5 mm Hg). We cannot exclude the possibility that the differences between the melting points are due to different procedures of determination; moreover a micro-melting point may differ appreciably from a macro-melting point.

Summary

One of the impurities seen in spectrophotometric analyses for commonly occurring alkaloids has been identified as tryptamine. A method has been devised for separating it from commonly occurring alkaloids during forensic chemical analyses.

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Acta pharmacol et toxicol 1965 22, 131–134

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Further Investigations into the Protective Action of Anaesthetics Against Anoxia in Mice

By

Bendt J Wilhjelm

(Received September 1, 1964)

In a previous publication (WILHJELM & ARNFRED 1965) the results are reported of an investigation into the protective action of inhalation anaesthetics on mice exposed to anoxia.

A significantly increased tolerance of mice to anoxia was found for tiomebumal, halothane and cyclopropane. Chloroform, trichlorethylene and methoxyflurane were seen to have a moderate action, but no action was observed with ether or nitrous oxide.

In continuation of these investigations we have estimated the protective action of some injectable anaesthetics on mice exposed to anoxia.

This study includes urethan, an anaesthetic frequently used in laboratories, and two more recently introduced anaesthetics hydroxydione = hydroxydion (WHO NFN) as sodium succinate (= viadril ®) and 2 methoxy-allylphenoxyacetic acid-N N-diethyl-amide (= detrovel ® = G 29 505). Use of the latter is still in the experimental stage. Further we experimented with anaesthetisation of mice with a mixture of pentothal = Thiopenthone = tiomebumal (NFN) and halothane = halotan (NFN WHO), the two anaesthetics previously shown to be the most active in increasing the tolerance of mice to anoxia.

ARNFRED & SECHER (1962) showed tiopental to have a considerable protective action against anoxia in mice. The results of this investigation have since been borne out, and a similar action on the tolerance of mice to anoxia has been found for halothane and cyclopropane (WILHJELM & ARNFRED 1965). Studies on hydroxydione and detrovel ® are not available, and it is a well-known fact that urethan inhibits cell-division, apparently without influencing oxygen uptake.

Table 1

Table showing the anaesthetic doses given in milligrams per animal and the time to induction f sleep.

Anaesthetic	Injected amount of anaesthetic in mg per animal	Time from injection to induced sleep in min.
Urethan	50 (i.p.)	2–3
Hydroxydione = hydroxydion (NFN)	1 (i.p.)	2–3
Detrovel ®	4 (i.p.)	1–2
Thiopentone = tiomebumal (NFN)	1.5–1 8 (i.v.)	½

Hydroxydione is the first steroid to have acquired some clinical use as an anaesthetic. In man hydroxydione reduces cerebral blood flow cerebral oxygen uptake and cerebral glucose metabolism (GORDAN, G. S., N GUADAGNI, J PICCHI & J E. ADAMS 1956) The reduction is of the same order as that seen after administration of a barbiturate.

Methods

As experimental animals we used male white mice ranging in weight from 25 to 30 g.

The experimental procedure and the anaesthetising apparatus employed have been described previously (WILHJELM & ARNFRED 1964)

Urethane, hydroxydione, and detrovel were injected intraperitoneally thiopentone was injected intravenously The doses given and the time elapsing before occurrence of sleep are shown in table 1

When asleep, the animals were placed in the bottles contained in the thermostat. During the experiments with urethan, hydroxydione, and detrovel the bottles were perfused with 20 % oxygen and 80 % nitrogen for ten minutes after the mice had been placed in them. Then the oxygen concentration was reduced to 5%, the nitrogen percentage being raised correspondingly During the experiments on anaesthesia with a combination of thiopenthone and halothane the bottles were perfused with a mixture of 1 % halothane, 20 % oxygen, and 79 % nitrogen for ten minutes. Then the oxygen concentration was reduced to 5%, the nitrogen concentration being raised correspondingly The halothane concentration remained at 1 % throughout the experiment. Percentages are v/v

The action of an anaesthetic was estimated by examining 100 mice, 50 anaesthetised and 50 controls. *The experiments were conducted as five pairs of duplicate.* Each of these involved ten mice exposed to anoxia under anaesthesia and ten non-anaesthetised but exposed to the same low oxygen concentration.

The survival time, i.e. the interval between reduction of the oxygen concentration to 5 % and the end of respiration, was measured for each mouse in both groups.

Results

Fig. 1 illustrates the distribution of the mice anaesthetised with the various *anaesthetics and those in the corresponding* control groups. *It is* seen that the anaesthetics used tended to prolong the survival of the animals exposed to anoxia while under anaesthesia.

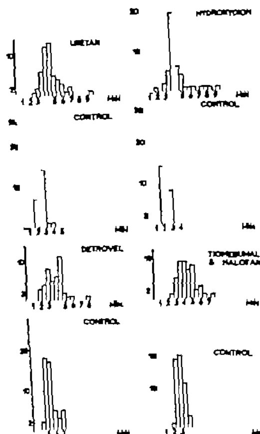


Fig. 1 Graphic representation of survival time for anaesthetised and non-anaesthetised mice exposed to anoxia. Abscissa survival time. Ordinate number of animals.

For the particularly long survivals we must, as in previous investigations, consider the possibility that leakage of the mouse bottles had caused the oxygen concentration to exceed 5 %. However even if we ruled out these values, we still find a marked prolongation of survival in anaesthetised animals.

The average prolongation of the survival time and its percentage prolongation are recorded in table 2.

Discussion

The investigations showed that all the anaesthetics tested prolonged the survival of mice exposed to anoxia, compared with survival times for non-anaesthetised anoxiated mice. A statistical analysis of the material showed a significant prolongation of survival time with all four anaesthetics ($p < 0.01$).

Table 2

Average survival time in minutes for control mice and anaesthetised mice, with percentage prolongation of survival time.
(In calculating the average survival time in table 2, extremely high values were excluded)

Anaesthetic	Average survival of control mice (min.)	Average survival of anaesthetised mice (min.)	*Increase of survival time (%)*
Urethan.	2.59	4.62	78.5
Hydroxydione = hydroxydion (NFN)	2.44	4.53	85.4
Detrovel ®	2.54	3.79	49.3
Thiopentone = Tiomebumal (NFN) and *Halothane (NFN,WHO)*	2.98	4.67	56.7

The prolongation of survival time that must be due to prevention of death in convulsions of anaesthetised anoxiated mice is, according to the results of previous studies (WILHJELM & ARNFRED 1964) considered to range between 8 and 12 %. The survival times measured in the material under review were, however so long as to render likely a genuine protective action of the anaesthetics against anoxia, beyond their mere anticonvulsive effect.

It has been shown previously (WILHJELM & ARNFRED 1965) that thiopenthone and halothane, given separately afford an appreciable protection of mice against anoxia. Anaesthetisation with a combination of thiopentone and halothane likewise protected in some measure against anoxia, but not to the same extent as the individual anaesthetics used alone

We cannot decide from these experiments whether or not the protective actions are of the same nature.

Summary

A protective action against anoxia has been demonstrated for urethan, hydroxydion and detrovel.

A combinaton of halothane and thiopenthone has been shown to have a corresponding protective action.

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Effect of Analgesics in Rabbits on Erythrocyte Survival Time.

By

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(Received September 9 1964)

It has been demonstrated by several investigators (FRIIS *et al* 1960 GSELL *et al.* 1957 LORENZEN & SCHWARTZ 1960 MIESCHER & PLETSCHER 1958 NIELSEN & FRIIS 1961) that exposure to phenacetin of patients suffering from extended abuse of this drug increases red-cell destruction manifesting itself in a reduced red-cell survival time.

Animal experiments have given similar results (MIESCHER & PLETSCHER 1958 PFLEGER *et al* 1961 PLETSCHER *et al* 1958) PLETSCHER *et al* (1958) found the red-cell survival time to be reduced more by the metabolites of phenacetin, N-acetyl-p-aminophenol and p-phenetidin, than by phenacetin. PFLEGER *et al* (1961) found a shortened red-cell survival in rats given aminopyrine.

It is not known whether a correlation exists between the ability of phenacetin to reduce the red cell survival time and its supposed damaging effect on the kidneys. There is reason to assume that the renal damage is non-specific, as human provocation tests (CLAUSEN & HARVALD 1961) have shown several commonly used analgesics to exert an irritating effect on the kidneys, as estimated by counting of urinary sediment, and animal experiments (CLAUSEN 1962 & 1964) have revealed histological renal changes after long-continued administration of phenacetin as well as of acetylsalicylic acid

If the renal damage and the red cell destruction are due to the same mechanism, it might be expected that analgesics other than phenacetin, for instance acetylsalicylic acid, would be able to reduce red-cell survival.

Material and Method

A total of 48 female rabbits, weighing about 2.5 kg each, were divided into 4 groups of 12. The drugs investigated were phenacetin, acetylsalicylic acid, N-acetyl-p-amino-phenol and a placebo. Each rabbit recieved 1.5 g of one of the substances named daily on 5 days a week for 12 weeks. The drugs were administered as tablets. The phenacetin and acetylsalicylic acid tablets were prepared in conformity with the Danish pharmacopoeia. The N-acetyl-p-aminophenol tablets were made for our particular purpose[1]) The placebo tablets were made of potato starch and lactose. During the experimental period two rabbits in the acetylsalicylic acid group died. After 6 weeks 4 ml blood were drawn from an ear vein of each rabbit. The red cells from each sample were labelled with about 25 mC $Na_2\,^{51}CrO_4$ (GRAY & STERLING 1950), and after being washed four times in physiological saline were re-injected into the same rabbit. Fifteen minutes later the first blood sample was removed, and during the following three days blood samples of 2 ml were removed daily and thereafter twice weekly during the subsequent 5–6 weeks.

The samples were submitted to haematocrit determination and measurement of the radioactivity All samples from each rabbit were counted at the same time in a well-type scintillation crystal detector with a conventional scaler

Results

For each rabbit the logarithm of the radioactivity (expressed as counts/min. corrected by means of the haematocrit value) was related to the time of blood sampling. The regression of the individual red-cell counts on time was calculated by the method of least squares.

Table 1 shows the mean values of the slopes of the regression lines for each group examined. It is seen that the rate of disappearance of labelled rabbit red-cells in the groups treated with phenacetin and acetyl-salicylic acid is significantly greater than that for the placebo group

Table 1

The mean values and their standard errors of the slopes of the regression lines for the red cell survival curve. Each group consists of 12 rabbits. The significance of the differences of the means for the placebo group and the groups treated with analgesics were calculated by the "t" test.

Group	$\Delta \log_{10} C/\Delta t$ Mean	s.e.m.	P
Placebo	−0.0268	0.0008	
Phenacetin	−0.0320	0.0013	<0.01
Acetylsalicylic acid	−0.0328	0.0015	<0.01
N-acetyl-p-aminophenol	−0.0271	0.0009	>0.40

1) Kindly supplied by Messrs. Dumex Copenhagen.



Fig. 1 Red-cell disappearance curve for the placebo group compared with the phenacetin group.
Abscissa time in days. Ordinate Activity as % of the 15-minute value on a logarithmic scale.



Fig. 2. Red-cell disappearance curve for the placebo group compared with the acetylsalicylic acid group.
Abscissa time in days. Ordinate Activity as % of the 15-minute value on logarithmic scale.

($p < 0.01$), whereas no significant difference was found between the placebo group and the group treated with N-acetyl-p-aminophenol. The average rate of disappearance per day was, for the placebo group 6.4 % for the phenacetin group 7.6 % for the acetylsalicylic acid group 7.8 % and for the N acetyl-p-aminophenol group 6.4 %.

This is shown graphically in figs. 1 2 and 3, where the average disappearance curve for the rabbits red cell in the placebo group is compared with those for the three treated groups. In each figure the radioactivity of the samples is given as a percentage of that at 15 minutes.

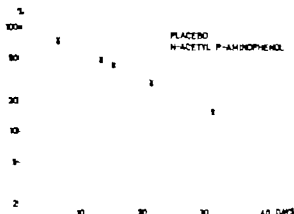


Fig. 3 Red-cell disappearance curve for the placebo group compared with the N-acetyl-p-aminophenol group. Abscissa time in days. Ordinat Activity as % of the 15-minute value on a logarithmic scale.

Discussion

PFLEGER *et al* (1961) found a reduced erythrocyte survival time in rats treated with phenacetin or with aminopyrine, but not after treatment with acetylsalicylic acid or N-acetyl-p-aminophenol.

PLETSCHER *et al* (1958) reported a reduced erythrocyte survival time in rabbits treated with N-acetyl-p-aminophenol or with p-phenetidin. In our study the erythrocyte survival time was significantly lower in the groups that received phenacetin or acetylsalicylic acid, whereas treatment with N-acetyl-p-aminophenol did not alter the erythrocyte survival time. Thus, the investigations performed so far into the effect of analgesics upon the erythrocyte survival time have not given consistent results, leaving the impression that several different analgesics may reduce the erythrocyte survival time in a way suggesting – as in renal damage – a non-specific phenomenon. This impression is also supported by the results of a previous experiment on rabbits (CLAUSEN & ANDRÉE LARSEN 1963, unpublished) which included, in addition to assessment of the substances tested in the study reported here, also involved a study of the effect of acetanilid, phenylbutazone and propyphenazone upon the erythrocyte survival time As compared with that of the placebo group, the erythrocyte survival time was significantly reduced during treatment with acetylsalicylic acid, slightly but not significantly reduced during treatment with phenacetin, acetanilid, phenylbutazone and propyphenazone, and almost unchanged during treatment with N-acetyl-p-aminophenol. However the groups were small comprising only 3–5 rabbits each, and it was considered that conclusions should not be based on that study alone

The results of these two investigations together now indicate that acetylsalicylic acid reduces erythrocyte survival time to the same extent as phenacetin, whereas the metabolic product of phenacetin, N-acetyl-p-aminophenol, does not affect it.

Two types of haemolytic anaemia have been described after intake of phenacetin. One is an acute variety occurring after intake of a single dose This variety occurs in the presence of a deficiency of the enzyme glucose 6-phosphate dehydrogenase. The other variety is chronic, manifesting itself in persons having had an excessive and long-continued consumption of phenacetin the mechanism of its onset is unknown, although an allergic mechanism has been suggested (FRIIS *et al* 1960 NISSEN & FRIIS 1961), and enzymatic as well as direct toxic mechanisms have been considered (LORENZEN 1962).

The mechanism of the damaging effect of analgesics upon the kidneys is not fully understood, but the histological renal changes observed in animal experiments involving administration of large doses of phenacetin or acetylsalicylic acid to rabbits over a long period (CLAUSEN 1962 & 1964) appear to indicate a direct damage to the tubular epithelium. On the other hand, none of the histological findings support the suspicion of allergy

It seems reasonable to assume that a corresponding, direct toxic action upon the erythrocytes is responsible for the erythrocyte destruction. Such toxic damage might well be caused by an inhibition of enzymatic reactions in the cells.

Summary

The erythrocyte survival time was studied on 48 female rabbits during treatment with phenacetin, acetylsalicylic acid, N acetyl-p-aminophenol and a placebo control. The erythrocyte survival time proved significantly shorter in the phenacetin and acetylsalicylic acid groups than in the control group, and no reduction was found in the N-acetyl-p-aminophenol group. Considered along with those of other investigations into the ability of various analgesics to reduce erythrocyte survival time, the results indicate that the erythrocyte destruction is a non-specific phenomenon.

Since this also appears to apply to the damaging effect of analgesics upon the kidneys- and since there appears to be some question of direct toxic action upon the tubular epithelium – it seems reasonable to assume that the erythrocyte destruction also is caused by a direct toxic action upon the erythrocytes.

Acknowledgement.

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Studies on the Fate of Dicoumarol-^{14}C in the Rat.

By

Flemming Christensen

(Received September 9 1964)

In an earlier paper we reported a study of the distribution and elimination of dicoumarol in rats after intravenous administration (CHRISTENSEN 1964a). The rate of disappearance of the drug from the organism followed the rate equation of a first order reaction, but only small amounts of the unchanged drug appeared in the urine and faeces. These facts indicate a transformation of the drug in the organism to metabolites, whose chemical nature is at present unknown. HAUSNER *et al.* (1951) have shown that feeding ^{14}C-labelled dicoumarol to rats results in the excretion of appreciable amounts of radioactivity in the urine and demonstrated that this activity represents several different radioactive compounds.

In order to obtain further information about the routes by which the metabolites are excreted from rats, we decided to study the problem by the use of ^{14}C labelled dicoumarol. This compound also enabled us to study the metabolites produced from dicoumarol and to establish their patterns in urine, faeces and tissues. Finally it was found possible to characterize and measure the amounts of some of the most important of them.

Material and Methods

Unlabelled dicoumarol (NFN) was obtained from a commercial source and used as a reference compound.

Dicoumarol labelled with ^{14}C in the methylene bridge (3,3'-methylene-^{14}C-bis (4-hydroxycoumarin)) was prepared by the method of LEE *et al.* (1950a). Paraformaldehyde-^{14}C, with a specific activity of 2 mc/mmol, was obtained from The Radiochemical Centre, Amersham, England, and converted to formaldehyde-^{14}C by maintaining it at 100° with water in a sealed tube for 2 hours. Formaldehyde-^{14}C was then condensed at room temperature with 4-hydroxycoumarin (Fluka, purum) to obtain labelled dicoumarol, which was recrystallised from boiling cyclohexanone. The specific activity of the labelled dicoumarol should be 2 mc/mmol.

The radiochemical purity of the drug was assessed by paper chromatography in the systems 1 2, and 3 mentioned below only one radioactive spot with an R_F-value *the same as that of the* reference dicoumarol being detected in each case. The radioactive compound and the authentic dicoumarol had the same melting point, and their molar absorbancies, determined at 314 mμ with 2.5 M NaOH as solvent, were practically identical.

The specific radioactivity of the labelled dicoumarol was 6.00×10^5 cpm/min/mg when counted at standard thickness, as described below In some of the experiments unlabelled dicoumarol was added to the labelled compound in order to reduce the activity

Animal experiments

White female rats from our animal stock, weighing 190–260 g, were fed on a commercial chicken diet and had free access to food and water during the experiments.

At the beginning of the experiment, normal rats were injected intravenously into the tail veins with a solution of the labelled dicoumarol prepared immediately before use by dissolving the compound in aqueous o 1. M NaOH. The animals were placed in metabolic cages for the separate and quantitative collection of urine and faeces and having a special device to avoid contamination with food. Urine and faeces were *collected for 24-hour periods during a total of 5 days.*

Because large amounts of radioactivity were excreted in the faeces of the normal rats, the possibility of excretion through the bile into the intestine was studied. Therefore the common bile duct was ligated in a series of rats under ether anaesthesia, by placing two ligatures around it one near to the porta hepatis and the other near the duodenum, cutting the duct between them. After three days two of the rats were selected for further experiments, both rats being in good general condition and exhibiting jaundice with dark urine. The experiments were then conducted as described for the normal animals.

Two rats were injected intravenously with ^{14}C-labelled dicoumarol and killed by heart puncture 6 and 24 hours after the injection. The liver and kidneys were removed and homogenised with 0.9 / sodium chloride. Extracts of these tissues and of plasma were prepared as described for urine and then submitted to paper chromatography.

Faeces collected from the individual periods were dried at 85 to a constant weight, usually reached in 2 to 4 hours, and then finely ground in a mortar All urine specimens were brought to a volume of 10 ml either by adding water or by evaporation under reduced pressure.

The radioactivity of the samples was measured by means of a Geiger Müller tube connected to a Berkeley decimal scaler All the activities recorded were corrected for the background count and then converted to the *activity at standard thickness*, to be defined below Dissolved radioactive materials were pipetted into aluminium planchettes (diameter 1 4 cm) mounted with a disk of lens tissue and glucose (25 μl of a 10 / (w/v) solution) as an adhesive. The samples were dried at 85° weighed and, if less than 0.2 mg of material was deposited on the planchet, referred to as having standard thickness. All activities recorded were converted to this standard condition for samples containing more than 0.2 mg this was done by means of a self-absorption correction curve obtained from experimental values. In assaying the radioactivity of faeces a special technique was employed, the finely ground material being suspended in a small amount of 0 1 M NaOH (10 μl/100 mg faeces) and water being added until a suitable consistency was obtained. The material was then transferred to an alumi-

alum planchette, 3–4 drops of water were added, and a uniform spreading of the sample was effected by means of a spatula. After drying at 85° for half an hour the sample was weighed and counted. The material deposited on the planchette should be about 40–50 mg, corresponding to an infinitely thick layer. The radioactivity measured was then converted to that of standard thickness by means of an experimentally determined correction factor.

With a few exceptions the counting error was kept to less than ±3 %. As a rule duplicate determinations of radioactivity were performed, but faeces samples were run in triplicate. The individual determinations on a sample seldom deviated more than a few % from the mean value, which is given as the result.

Extracts of urine were obtained by shaking the acidified samples, saturated with solid NaCl, with 5 vol. of ethyl acetate for 5 min. and repeating this procedure 3 times. The mean radioactivity of the combined extracts, which were evaporated to dryness in vacuo, amounted to 92 % of that present in the original sample. Known fractions of the extracts were submitted to one- and two-dimensional paper chromatography. Extracts of plasma and tissue homogenates were prepared in essentially the same way as those of urine and also submitted to chromatography.

Extracts of faeces, which could not be satisfactorily obtained by the method used for urine, were prepared differently. The finely ground material (250 mg) was placed in a glass-stoppered flask containing 5 ml of 1 M HCl and 40 ml of pure acetone. The flask was vigorously shaken mechanically for 3 hours, the contents then being filtered and the filtrate brought to a volume of 50 ml by adding pure acetone. The mean radioactivity of the total filtrate amounted to 88 % of that present in the original sample. However, the extract was too impure to be used directly for paper chromatography, and the filtrate was concentrated under reduced pressure to a small volume (5 ml), solid sodium chloride being then added to saturation and the concentrated filtrate extracted 4 times with 2 vol. of ethyl acetate. The combined extracts were evaporated to dryness under reduced pressure, and the residue was dissolved in 5 ml of 2.5 M NaOH and then extracted with one volume of ethyl acetate, which was removed and discarded. The sodium hydroxide was acidified with conc. HCl and then extracted 3 times with 2 vol. of ethyl acetate, the total extracts then being evaporated to dryness under reduced pressure. The residue, whose mean total radioactivity amounted to 65 % of the original, was then submitted to paper chromatography.

It is to be noted that paper chromatographic examination of urine and faeces was performed only on samples obtained for the first 24-hours period of each experiment.

The paper chromatographic procedure was as previously described (CHRISTENSEN 1964b), the solvent systems used for the development of the chromatograms being:

System 1 n-butanol – 3 M-aqueous ammonia 1:1 (v/v)
System 2 Benzene – acetic acid – water 2:2:1 (v/v)
System 3 Ethyl acetate – 3 M-aqueous ammonia 1:1 (v/v)

One-dimensional paper chromatograms were usually developed by system 1, and two-dimensional paper chromatograms by system 1 and 2 for the first and second directions, respectively.

The colour reactions employed for detecting the metabolites on the chromatograms were those of diazotized sulphanilic acid and diazotized o-dianisidine, as modified by CHRISTENSEN (1964b), the coupling being carried out at about pH 7 in all cases.

Autoradiographs of 2-dimensional paper chromatograms were prepared by placing them in contact with a Ferrania x-ray film (type N, blue base) for at least 8 days.

One-dimensional paper chromatograms were scanned by means of an automatic windowless paper chromatogram scanner (model RSC-5, Atomic accessories Inc.,

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N.Y.). The total area enclosed by the resulting curve, as well as the areas of the individual peaks, were determined by cutting them from the record and weighing the paper pieces, thus permitting an estimate of the relative distribution of the radioactivity on the individual spots.

Results

The excretions of radioactivity in urine and faeces by normal rats after the intravenous administration of dicoumarol labelled with ^{14}C are shown in table 1. The mean value of the total radioactivity excreted by the 3 rats during the 5 days of the experiments amounted to 94.0 % of the injected dose, the greater part being found in the faeces (mean value 71.3 %) and the smaller (mean value 22.8 %) in the urine. During the first 24-hours period an average of 75.0 % of the activity appeared in the excreta, this percentage being reduced to 13.7 during the next 24-hours period. During the last 24-hours period the percentage had decreased to 0.7.

Table 1

Excretion of radioactivity in urine and faeces by normal rats after intravenous injection of dicoumarol labelled with ^{14}C.

Exp. No.	Dose of dicoumarol-^{14}C	Period (hours after injection)	Activity recovered (% of dose) urine	faeces	total
1	4.70 mg ~ 2.82 × 10^6 cpm	0–24	17.7	63.7	81.4
		24–48	2.5	8.0	10.5
		48–72	1.1	1.8	2.9
		72–96	0.3	1.0	1.3
		96–120	0.1	0.4	0.5
			Total 21.7	74.9	96.6
2	4.30 mg ~ 2.58 × 10^6 cpm	0–24	15.6	50.0	65.6
		24–48	3.5	11.6	15.1
		48–72	1.0	2.8	3.8
		72–96	0.6	0.8	1.4
		96–120	0.2	0.5	0.7
			Total 20.9	65.7	86.6
3	4.81 mg ~ 1.38 × 10^6 cpm	0–24	19.9	58.0	77.9
		24–48	3.1	12.3	15.4
		48–72	1.5	1.8	3.3
		72–96	0.7	0.6	1.3
		96–120	0.5	0.5	1.0
			Total: 25.7	73.2	98.9

The excretions of radioactivity in urine and faeces by rats with ligature of the common bile duct after the intravenous injection of labelled dicoumarol is shown in table 2. Because of extensive hemorrhages into the peritoneum, etc., the rats died between the third and fourth day after the injection, allowing only three collections from each rat. The total excretion of radioactivity in the excreta during the experiments was 82.1 and 91 7 %, giving an average value of 86.9 %. The excretion of radioactivity in the faeces was, however low and the mean value for the excretion by this route during the 3 days was only 4 4 %. Also in these experiments the greater part of the total activity was excreted during the first 24-hours (mean value 69 6 %), and during the following 24-hours the excretion decreased to a mean value of 13 7 %.

Table 2

Excretion of radioactivity in urine and faeces by two rats with artificial occlusion of the common bile duct after intravenous injection of dicoumarol labelled with ^{14}C.

Exp. No.	Dose of dicoumarol-^{14}C	Period (hours after injection)	Activity recovered (% of dose) urine	faeces	total
4	5.33 mg ~ 3.32 × 10^6 cpm	0–24	64.3	2.9	67.2
		24–48	9 7	3 7	13.4
		48–72	1.5	–	1.5
			Total 75.5	6.6	82.1
5	4.04 mg ~ 1.21 10^6 cpm	0–24	70.3	1.6	71 9
		24–48	13.4	0.5	13.9
		48–72	5.9		5.9
			Total 89.6	2.1	91 7

The patterns of radioactive metabolites in urine and faeces as revealed by one- and especially by two-dimensional paper chromatography were somewhat complex, as an extensive spread of the radioactivity of many different compounds was invariably noted. This is illustrated by fig. 1 showing a typical autoradiograph of a two-dimensional paper chromatogram from faeces. In order to facilitate the further characterization of the individual radioactive compounds, we designated each of these from the faeces by a letter and also by a number referring to the R_f-value of the compound chromatographed by system no 1 (butanol – aqueous ammonia). In this way some of the most important radioactive metabolites in

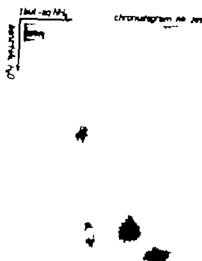


Fig. 1 Typical autoradiograph of 2-dimensional paper chromatogram for faeces of the rat after intravenous administration of dicoumarol labelled with ^{14}C. The relative positions of the main radioactive compounds can be seen.

faeces were designated, as can be seen from table 3 but those radioactive compounds having low R_f-values (< 0.20) in both systems 1 and 2 are excluded, as they could not be clearly separated and characterized by the methods employed Likewise compounds represented by weak and variable spots on the chromatograms are omitted. Further table 3 shows the R_f-values of the individual metabolites and their colour reactions with diazotized sulphanilic acid and diazotized o-dianisidine. The R_f-values given in the table were determined by submitting an extract of faeces to paper chromatography by system 1 eluting the individual radioactive zones from the chromatogram and finally submitting the eluates to chromatography with each of the 3 solvent systems used In this way the R_f-values obtained were somewhat higher than those obtained with crude extracts of faeces, indicating that impurities may reduce the R_f-values of the compounds.

The pattern of radioactive metabolites in urine resembled somewhat that for the faeces, but the compound D_2-032 was absent from the urine and the compound A-078 only present in small amounts (table 3). The other compounds detected in the urine, except those with low R_f-values (< 0 20) not considered, were apparently identical with those found in the faeces The assumed identity of a compound in urine with that in faeces was assured as far as possible by again chromatographing the two compounds side by side, on the same papers, with each of the 3 solvent systems described for the development.

Table 3

Some important radioactive metabolites detected in the urine and faeces of rats after the intravenous injection of dicoumarol-14C. R_F-values of the individual compounds as well as colours obtained with the colour reactions employed are shown. A rough estimat of their relative abundances is also given. The corresponding properties of dicoumarol are shown for comparison.

Compounds	R_F-values¹)			Colours		Relative amounts²)	
	system 1	system 2	system 3	diazotized sulphanilic acid	diazotized o-dianisidine	urine	faeces
Dicoumarol	0.78	0.95	0.35	yellow	purple		
A 078	0.78	0.94	0.35	yellow	purple	(+)	++
B 055	0.55	0.82	0.11	yellow	purple	+++	+++
C 040	0.40	0.87	0.03	yellow	purple	++	+
D 034	0.34	0.79	0.03	yellow	purple	0	+
D_1 032	0.32	0.38	0.03	yellow	purple	++	++
(O)	0.06	0.02	0	?	?	++	?

¹) Solvent systems
System 1 n-butanol-3 M-aqueous ammonia 1 1 (v/v)
System 2 Benzene-acetic acid-H_2O 2 2 1 (v/v)
System 3 Ethyl acetate-3 M-aqueous ammonia 1 1 (v/v)
²) The number of + indicates the relative amounts present.

The compound A-078 was, from its R_F-values and colour reactions, indistinguishable from dicoumarol (table 3), but the chemical nature of the other metabolites is entirely unknown.

Table 4 shows the distribution of radioactivity isolated from urine and faeces during the first 24-hours after injection of labelled dicoumarol, for some of the metabolites previously mentioned. The distribution was obtained by scanning one-dimensional paper chromatograms as described

Table 4

The percentage distribution of radioactivity in various radioactive compounds in urine and faeces obtained from the period 0-24 hours after intravenous injection of 14C-labelled dicoumarol into rats. Extracts of urine and faeces were submitted to one-dimensional paper chromatography and the chromatograms were scanned to obtain the relative activities. The mean values given for the individual compounds were determined from duplicate determinations on 3 different animals. The ranges are shown in parenthesis.

Compounds	Relative amounts (%) urine	Relative amounts (%) faeces
A-078 (dicoumarol)	7.3 (4-10)	16.6 (13-20)
B-055	35.5 (27-48)	37.0 (33-40)
Others	56.5 (42-66)	46.4 (43-50)

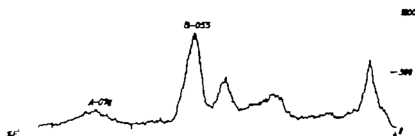


Fig. 2. Typical record obtained by scanning a one-dimensional paper chromatogram prepared from the urine of rat previously injected with ^{14}C-labelled dicoumarol. The chromatogram was developed by system 1 (butanol – aqueous ammonia).
S indicates point at which the material was applied to the chromatogram and SF the position of the solvent front after development. The ordinate shows the activities recorded in cpm.

under Methods. A typical scanning record for urine is shown in fig. 2. The single compound accounting for most of the radioactivity was evidently B-055.

The patterns of radioactive compounds in the plasma and liver after intravenous injection of dicoumarol-^{14}C, as revealed by two-dimensional chromatography, showed the presence of unchanged dicoumarol as well as at least two radioactive metabolites, the dominant one apparently being identical with the metabolite B-055 from faeces and urine, the other running with low R_f-values by both system 1 and 2. The relative distribution of radioactivity among these various compounds, as obtained by scanning one-dimensional chromatograms, is shown in table 5. At 6 hours after the injection of dicoumarol-^{14}C the unchanged compound accounted for the larger part of the total activity present in both the plasma and the liver (55 and 79 % respectively), but after 24 hours the metabolites accounted for 55 % of the activity in plasma and 82 % of that in the liver.

Table 5

The percentage distribution of radioactivity in various compounds detected in the livers and plasmas of rats at 6 and 24 hours after intravenous administration of dicoumarol-^{14}C. Extracts of plasmas and livers were submitted to paper chromatography and the chromatograms were scanned to obtain the relative activities shown.

Compounds	Liver (%)		Plasma (%)	
	6 hours	24 hours	6 hours	24 hours
Dicoumarol	55	19	79	46
Metabolite B-055	77	35	15	24
Other metabolites	17	47	4	31

Discussion

The results presented in this paper indicate that dicoumarol in rats is *predominantly eliminated* as metabolites, which are excreted together with small amounts of the unchanged drug in urine and faeces. In the studies by HAUSNER *et al* (1951) on rats, it was shown that no radioactivity appeared in the expired air after oral administration of dicoumarol labelled with ^{14}C in the methylene bridge this is similar to the finding, of LEE *et al* (1950b) on mice after the intravenous administration of the radioactive compound We have in our study therefore not taken this possible route of elimination into consideration.

From the results it is also apparent that most of the dicoumarol and its metabolites are excreted in the faeces and only a minor part in the urine The results of ligating the common bile duct indicate that the metabolites reach the intestine with the bile. In their studies LEE *et al* found a similar distribution of radioactivity in urine and faeces after the intravenous injection of ^{14}C-labelled dicoumarol into mice, but in corresponding experiments on rabbits only a small fraction of the activity appeared in the faeces. A pronounced species difference thus apparently exists. LEE *et al* also suggested that there is excretion of metabolites through the bile especially as they found the concentration of radioactivity in this fluid to be high. HAUSNER *et al* have reported a preliminary experiment on the excretion of radioactivity in urine and faeces of rats during the first 24-hours after injection of ^{14}C-labelled dicoumarol. A total of 48.4 % of the dose appeared in these excreta, most (32.6 %) being found in the faeces, including the gastrointestinal tract with its content, and the smaller part (15.8 %) in the urine. These figures agree well with our own findings, although the total excretion during the first 24-hours in our experiments, amounting to a mean value of 75.0 %, was somewhat higher than in theirs.

In a previous paper (CHRISTENSEN 1964a) we reported the rate of disappearance of dicoumarol from the rat, the half-life being found to be 7.6 hours. If this be compared with the rate of disappearance of radioactivity from the rats, which is easily obtained from the results of the investigation now described, it is evident that with increasing time after injection of dicoumarol labelled with ^{14}C the total radioactivity remaining in the rats must represent a decreasing fraction of dicoumarol and an increasing fraction of its metabolites. This accords well with the figures given in table 4 and agrees with the increasing concentrations of the metabolite B-055 in plasma observed in our previous study (CHRISTENSEN 1964a) during the first 8–12 hours after the intravenous injection of dicoumarol.

The patterns of radioactive compounds in the urine and faeces were, as already stated, rather complex in order to simplify matters, weak and variable radioactive spots have been neglected Unchanged dicoumarol accounted for 10–15 / of the dose injected, but a much larger fraction was excreted as the metabolite B-055 these two compounds accounting for about one half of the total amount of radioactivity excreted during the first 24-hours.

As far as is known, little information about the metabolites produced from dicoumarol in man and animals is available, the most important contribution to our present knowledge on this matter being the studies of HAUSNER *et al* already referred to They succeeded in separating the radioactivity present in the urine and plasma of rats, given ^{14}C-labelled dicoumarol orally into several radioactive compounds by means of one-dimensional paper chromatography with methanol – butanol – water as the solvent, but the metabolites were not characterized As we did not use this solvent system, our results cannot be compared with those of HAUSNER *et al.*, but it is evident from both studies that dicoumarol in the rat is metabolized to several different compounds. Among these, the substance B-055 is quantitatively the most important one, consistently with our previous suggestion (CHRISTENSEN 1964a) The possibility that one or more of the radioactive compounds detected in the urine, faeces, etc. of rats might be formed by the action of intestinal bacteria should be considered, but evidence against this theory was obtained by demonstrating in the urine from rats with ligature of the common bile duct, the same metabolites (together with several new ones) as were found in the urine of normal rats. It would be expected that metabolites formed by intestinal bacteria would disappear from the urine under these conditions, when so little radioactivity apparently reached the intestine.

Further HAIS & PROHÁZKA (1954) have reported that feeding dicoumarol to rabbits results in the excretion in the urine of a metabolite running with an R_f-value of 0 50–0 55 in the system butanol-ammonia. This metabolite might be the same as that designated B-055 In human urine WEINER *et al* (1950) were unable to detect either dicoumarol or conjugates with glucuronic or sulphuric acids after the administration of dicoumarol. Thus it can be concluded that, although metabolites of dicoumarol have been demonstrated in some animal species, nothing is at present known about their structure.

In the plasma, at least up to 24-hours after the injection of dicoumarol labelled with ^{14}C, three radioactive compounds were detected in our study whereas HAUSNER *et al* reported three and a weak fourth. Likewise in the liver tissue we found 3 radioactive compounds, one being unchanged dicoumarol LEE *et al.*, however could only detect unchanged

dicoumarol in the livers of dicoumarol-^{14}C treated rabbits by the use of the isotope dilution method. These discordant results may be due to a species difference in the fate of dicoumarol.

Summary

The excretion of radioactivity in urine and faeces after the intravenous administration of dicoumarol labelled with ^{14}C to rats has been studied. Most of the activity was recovered from the faeces and a smaller amount from the urine, the total amount excreted during 5 days being nearly equal to that of the dose administered. Artifical occlusion of the common bile duct resulted in a reversal of this situation, practically all the activity being excreted in the urine and only small amounts in the faeces.

A specific pattern of radioactive metabolites in urine and faeces of the normal rats was established by means of paper chromatography. The most important of the metabolites were characterized by their R_f-values and colour reactions with diazotized sulphanilic acid and diazotized o-dianisidine. Quantitative estimation of some of the metabolites were also carried out.

The radioactive compounds in plasma and liver tissue were separated by paper chromatography into unchanged dicoumarol and metabolites. The main part of the radioactivity in the liver and plasma after 24-hours after the injection of dicoumarol-^{14}C, represented metabolites.

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The Effect of Pirinitramide (R 3365) on the Respiratory Response to CO_2 Inhalation

By

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Pirinitramide, (R 3365), (2,2-diphenyl-4-(4-piperidino-4-carbamoyl-piperidino)butyronitrile) is a new potent analgesic, which lacks some of the classical chemical features of the known morphine-like compounds. Pharmacological tests have shown it to have a number of typical morphine-like properties In most tests on mice rats and dogs, it is about twice as active as morphine by the subcutaneous route. In depressant effect on the respiratory rate of rats it was found not to differ significantly from morphine. Emetic activity in dogs is low (JANSSEN 1961) In a clinical trial (CERASO *et al* 1963), pirinitramide was used on 75 patients for the treatment of postoperative pain and found to provide excellent analgesia with few side effects.

With this background it was thought of interest to subject pirinitramide to an experimental investigation into its influence on respiration. In the study reported here such an investigation was undertaken by a technique adapted from a method described by ECKENHOFF *et al* 1956.

Method

Observations were made on four male subjects whose ages and body weights are shown in table 1 During an experiment, the subject was lying supine. He was fitted with a nose clip and breathed through a mouthpiece and a unidirectional valve in a "semiclosed" circuit, which incorporated a reservoir bag of 5.5 l capacity a pneumotachograph (Fleisch) and an exhaust valve for the escape of excess gas. A continuous adjustable flow of a mixture of 50 % oxygen and 50 nitrogen, metered in a flowmeter (rotameter), entered the circuit through the bag. The expired gas was sampled by continuous suction at a flow-rate of 2.5 l/min. from a point just distal to the expiratory part of the unidirectional valve, and the carbon dioxide concentration of the sample was continuously measured in an infrared CO_2 analyser (Capnograph, Godart) The

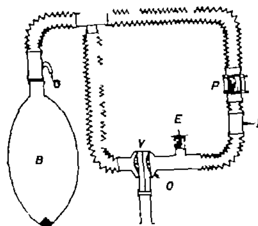


Fig. 1. The breathing circuit. V unidirectional valve; O, expired gas sampling. E, exhaust valve; I, re-entry into circuit of CO_2 analysed gas. P pneumotachograph. B reservoir bag. G inlet for oxygen-nitrogen mixture.

gas that had passed through the analyser was directed back into the circuit proximally to the pneumotachograph. The CO_2 / of the expired gas and the respiratory volume were recorded continuously on a Honeywell recorder. Expired volume was represented by a tracing, which rose in step with the respiratory rate. The amplifier of this tracing was zeroed at one-minute intervals, the height of the tracings thus indicating the respiratory minute volume. The volume of the circuit with the bag lightly distended was approximately 7.5 l. The breathing circuit and the position of the various parts incorporated in it are shown in fig. 1. A detailed description and an illustration of the recording arrangements are given by DYRBERG *et al.* (1962).

When a subject began breathing in the circuit, expired CO_2 accumulated in the system at a decreasing rate until a steady state was obtained. The carbon dioxide concentration in the end-tidal gas at which a steady state was reached depended on the volume of gas leaving the circuit, which again was equal to the volume of carbon dioxide-free gas admitted into it. In this manner it was possible to control the level of end-tidal carbon dioxide concentration by adjusting the rate of flow of carbon dioxide-free gas into the circuit. An end-tidal carbon dioxide concentration in the region of 6.5–7.0% was aimed at. The oxygen concentration of the inhaled mixture was about 40 . With the large ventilation produced by carbon dioxide inhalation the bag was nearly emptied at every inspiration, thus ensuring effective mixing of the inhaled gas.

After a control period of at least five minutes at a steady end-tidal CO_2 , the experimental drug was injected intravenously through a needle put in place before the beginning of the experiment proper. As a rule the flow of oxygen/nitrogen had to be re-adjusted until the end-tidal CO_2 was again steady for at least five minutes at the CO_2 level used as control. Respiratory minute volumes before and after the injection were determined as the averages of the minute volumes in the five minute periods of identical CO_2 % before and after the injection.

Either pethidine 50 mg/70 kg body weight, or pirinitramide, 15 mg/70 kg, was administered. The nature of the drug injected was not revealed to the experimental subject. Ten experiments in all were performed. Pethidine was given once to each subject, pirinitramide once to two subjects, and twice to the other two, as tabulated in table I. Intervals between experiments were at least 24 hours.

Results

The experimental result are given in table I

It is seen that the *respiratory minute volumes* were reduced in all four persons after the administration of either pethidine or piritramide. The reduction was more pronounced after piritramide than after pethidine in three of the four subjects, though in one the opposite was found. *Respiratory rate* was also generally reduced, with three exceptions, in two of which it was increased and in one unchanged. The relative reductions in respiratory rate and respiratory minute volume were almost the same after piritramide. After pethidine, the respiratory rate was reduced relatively less than the respiratory minute volume.

After the experiments with piritramide, all of the subjects stated that they had experienced one or more of certain sensations, which generally were of a pleasant nature euphoria a feeling of being less bothered by the forced breathing lightheadness a drunken feeling dizziness. After the experiments some degree of malaise, headache and a "peculiar feeling persisted for some hours. Similar subjective reactions were reported after pethidine, but were less pronounced

Discussion

The method used in this study has some theoretical advantages over that of our previous studies on drug-induced changes in the regulation of respiration. The main feature of our earlier method consisted in a series of total rebreathing procedures in a closed circuit, which allowed endogenous carbon dioxide to accumulate rapidly and "indefinitely". Changes in respiratory regulation were assessed from comparisons between respiratory minute volumes at an arbitrarily chosen elevated level (or several levels) of end tidal carbon-dioxide concentrations. With the modified method the subject is breathing uninterruptedly at a constantly elevated alveolar carbon dioxide concentration in a partial rebreathing system, and respiratory regulatory changes are assessed from changes in respiratory minute volume.

The purposes of this modification were 1) to make changes in the ventilation/end tidal P_{CO_2}-ratio more easily assessed, as it is possible to maintain end-tidal carbon dioxide concentration at a constant level thus making any changes in the above-mentioned ratio immediately apparent from the changes in respiratory minute volume 2) to make it possible to monitor and record end-tidal carbon dioxide concentration and respiratory minute volume continuously before, during and after administering the agent under study. This would make evaluation of the time-relations of the drug effects simpler and more accurate 3) to eliminate the problems

Table 1

Respiratory minute volume nd rate t elevated expired carbon dioxide concentration before and after intravenous injection of pirinitramide 15 mg/70 kg or pethidine 50 mg/70 kg. RMV = respiratory minute volume.

Drug	Exper subject			Date June	End-tidal CO_2 %	Before injection			After injection			Average exper results in per cent of control values	
		Age	Body weight			Control period, minutes before injection	RMV l/min.	Resp. rat	Exper period, minutes after injection	RMV l/min.	Resp. rat	RMV	Resp. rate
Pirinitramide	O.N	20	54	3	7.2	18	27.5	21	39	24.7	23	89.7	109.5
				17	6.7	5	23.9	24	10	23.5	21	90.8	87.5
	O.C.	17	64	9	6.9	5	39.2	43	20	37.3	28	63.2	65.0
				12	6.3	12	36.0	20	10	24.4	19	67.7	95.0
	J.M.	20	84	10	6.9	5	40.9	24	30	26.2	23	64.1	95.8
	J.N.	19	70	19	6.8	8	41.9	22	7	30.7	21	73.3	95.5
	Average				6.8		38.6	26		27.8	23	74.8	76.4
Pethidine	O.N			4	7.1	5	21.5	25	3	17.6	22	81.6	88.0
	O.C.			11	6.7	5	48.4	29	3	37.3	23	77.0	79.3
	J.M.			15	6.6	13	41.8	23	20	33.8	25	80.9	108.8
	J.N.			17	6.4	7	31.6	22	34	27.3	22	86.3	100.0
	Average				6.7		35.6	25		29.0	23	81.5	94.0

associated with the transition from breathing normal air to breathing carbon dioxide mixtures (e.g. changes in oxygen concentration of the alveolar gas, cardiac output, the pulmonary blood volume (SHEPHARD 1955), reaction time of the respiratory regulation in response to increased arterial carbon dioxide tension)

In most of the experiments the carbon dioxide concentration increased slightly immediately after injecting either drug, presumably because of a sudden reduction in pulmonary ventilation. This short elevation of carbon dioxide concentration was followed by a slow decrease in both the carbon dioxide concentration and respiratory minute volume towards a new steady state. The decreasing tendency of the end tidal carbon dioxide concentration was reversed by a reduction in flow of carbon dioxide free gas into the circuit in such a manner that end-tidal carbon dioxide concentration increased and reached the pre-injection level. These changes are illustrated in fig. 2a & b

The fall in end-tidal carbon dioxide concentration, which followed the injection of the drug, could be caused by a reduced carbon dioxide output from the experimental subject, such as would follow reduced ventilatory work. The reduction in carbon dioxide concentration could also be due

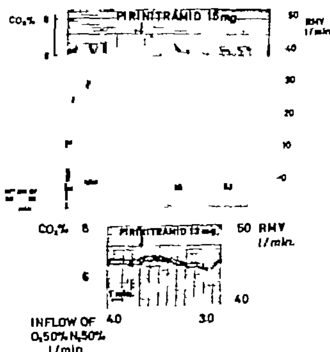


Fig. 2a and b. Tracings from two different experiments. The nearly horizontal tracings represent CO_2 % of expired gas, the interrupted nearly vertical tracings represent respiratory minute volumes (RMV).

to an increase in the amount of carbon dioxide eliminated from the circuit in consequence of a proportionately greater escape of alveolar gas than dead space gas from the exhaust valve. This possibility has not been investigated, but seems unlikely Because of the position of the exhaust valve close to the expiratory valve, and the fact that sufficient pressure to open the exhaust valve was only reached at the end of expiration, mainly and probably exclusively alveolar gas was eliminated from the circuit, irrespective of changes in ventilatory size and rate. Finally a decreased carbon dioxide concentration would result from an increase in the volume of gas in which the carbon dioxide is present. This volume consists of the mechanical dead space of the breathing circuit and the subject s airway and lungs. There is hardly any reason to suspect any of these volumes of changing under the conditions of the experiments described.

Consequently it seems reasonable to assume that the main cause for the reduced carbon dioxide concentration observed after the injection is the reduction in ventilatory work.

Owing to the relatively large volume of the breathing circuit, changes in carbon dioxide concentration in response to adjustments of the flow of carbon dioxide-free gas into the circuit occurred too slowly to allow sufficiently speedy control of this factor and it proved difficult to predict with adequate precision how much the flow of carbon dioxide-free gas into the circuit had to be altered for the experimental subject to reach the desired end-tidal carbon dioxide concentration. This inertia is inherent in the method, since the reservoir bag must be larger than the tidal volume of the subject. For this reason the time needed to reach the desired end tidal carbon dioxide-concentration varied greatly as is evident from the information given in table I In ability to provide a basis for a minute to-minute assessment of drug effects, this method has consequently proved less than optimal, although it is an improvement over our previous method.

Differences in the sensitivity of the present method and the *total* rebreathing method, referred to above, in gauging changes in the respiratory responses to carbon dioxide inhalation may be evaluated on comparing the results obtained by the two methods. In a study undertaken with the total rebreathing method (DYRBERG *et al* 1962), pethidine 50 mg/70 kg body weight was found to reduce pulmonary ventilation at elevated alveolar carbon dioxide concentrations in three experimental subjects by 24.8 %, 30.4 % and 32.4 %, i.e. greater changes than in the new study in which the average reduction in pulmonary ventilation of four experimental subjects, after an identical dose of pethidine, was found to be 18.5 %. These figures appear to suggest genuine difference in response by the two

methods. Since by the method based on a constantly elevated carbon dioxide concentration less ventilatory reduction is found than by the method in which ventilation is measured *on the slope of a rapidly increasing* carbon dioxide concentration curve, it may be assumed that, besides the reduced ventilation/P_{CO_2} ratio found by both methods, a delay in respiratory response is recorded by the total rebreathing method.

The reduction in ventilation/P_{CO_2} ratio found after injection of pirinitramide 15 mg was considerable, and on an average somewhat greater than that after pethidine 50 mg. Whether the difference between the two drugs is of practical significance is impossible to decide at the moment, because of lack of knowledge about the analgesic potency of pirinitramide. The scanty information available seems to suggest that 15 mg of pirinitramide has a somewhat stronger analgesic activity than 50 mg of pethidine. Provided this is so our study confirms the generally held belief that respiratory depressant and analgesic activity of the potent analgesics so far known run roughly parallel.

Finally this study has demonstrated that pirinitramide causes considerable depression in respiration. Besides, it was found capable of producing euphoria. No evidence was found to suggest that pirinitramide differs significantly from pethidine in these respects

Summary

The respiratory effects of pirinitramide have been compared with those of pethidine by an experimental arrangement for measuring the ratio between respiratory minute volume and end tidal carbon dioxide concentration during inhalation of carbon dioxide mixtures. Pirinitramide was found to possess a respiratory depressant activity of the same order of magnitude as pethidine The method of investigation is discussed.

Acknowledgement.

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Tolerance to Anticonvulsant Drugs

By

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Although there has accumulated much experimental and clinical evidence for the development of tolerance to the action of morphine-like analgesics, hypnotics and antitussives, there is no general agreement about the existence of tolerance to the action of anticonvulsant drugs.

This seems especially curious with phenobarbital, since the development of tolerance to the sedative and hypnotic effect of this drug is well known and has been studied extensively (KRAUTWALD & OETTEL 1937 BUTLER *et al* 1954 LOUS 1954 SUNSHINE 1957 REMMER *et al* 1960, 1962a & b). HENRIKSEN & KRISTIANSEN (1964), in their book on epilepsy note the development of tolerance to the sedative effect of the drug, but believe that this tolerance does not extend to the anticonvulsant effect.

About diphenylhydantoin, GOODMAN & GILMAN (1958) state "Rarely a slight degree of tolerance may develope and may necessitate an increase in dose more often the effectiveness of the drug appears to increase with time " STRAUSS (1959) notes the necessity sometimes to increase the dose of anti-epileptics after medication for some time however this specially applies to children and he considers it to be the consequence of natural growth under the treatment, with a consequent fall in the relative dose. JOVO (1964), reporting on clinical experience with a new synthetic product, states that "as with other antiepileptics" there was a tendency to reduced effectiveness after 2–4 weeks of treatment.

SCHMID *et al* (1964) found a tolerance to the sedative and anticonvulsant effects of glutethimide in rats after administration of the compound for 60 days, but they do not supply further experimental values for the phenomenon.

Against the background of this conflicting evidence for the existence of tolerance to anticonvulsant drugs, we decided to study the phenomenon experimentally for some representatives of this class, namely phenobarbital, diphenylhydantoin and ethosuccinimide Since reliable methods for determining anticonvulsant effects are available only for small laboratory animals, the study was performed on mice

Material and Methods

Mice of either sex, of the Leo strain, weighing 20-22 g, were used.

The anticonvulsants were given in the drinking water over periods of 1-14 days, the concentrations being chosen so as to permit the construction of anticonvulsant dose-effect curves. Each concentration was tested on a group of 20 mice, the sexes being kept in separate cages. The bottles containing drug solution were weighed every day and the daily intake in mg/kg was calculated. The drug solutions were prepared fresh every day The mice were weighed twice weekly in the experiments of 4-14 days duration.

After periods of 1 2, 4, 7 or 14 days, the anticonvulsant efficacy of the medication was determined by the maximal electroshock seizure test for phenobarbital and diphenylhydantoin or in the pentetrazole seizure threshold test for ethosuccinimide. Both tests were performed along the general lines described by SWINYARD *et al.* (1952) and have recently been described in detail (FREY 1964).

After having been tested for protection against electro- or chemoconvulsions, the mice were beheaded and the serum concentrations of the relevant drug were determined both for protected and unprotected mice and for the sexes seperately

Phenobarbital was extracted by a modification of the methods recommended by GOLDBAUM (1952) and BRODIE *et al.* (1950), as outlined in a previous paper (FREY *et al.* 1961), and determined spectrophotometrically the extinction difference between 240 and 275 nm being taken as a measure of the barbiturate concentration. Diphenylhydantoin was determined by the method of SVENSMARK & KRISTENSEN (1963), the only modification being that we extracted 1 ml serum with 20 ml chloroform. Ethosuccinimide was determined by the method of HANSEN (1963). The injected pentetrazole did not interfere with this method.

For each period of treatment the drug concentrations in the drinking water, the
serum concentrations and the average daily drug intakes ... ned against the
anticonvulsant effects, and the drug concentrations in ... the serum
concentrations (SC) and the average daily doses (DD) ... on were
determined by the graphical method of ... W ... The statistical
significance of the difference from the one ... the methods
described in the same paper All doses and ... of the
free acids.

Phenobarbital (phenemalum NFN) ... as in
sodium salt, diphenylhydantoin with ... a clear
solution of the latter it was necessary to
was given as a dilution with tap water
5 / of the drug (Zarondan-Saft ®). ... the

The half-life time of the anticonvu
analytical methods mentioned after intra
30 mg/kg diphenylhydantoin or 100 mg
compound in water). Groups of
and the drug concentrations in the

The sedative-hypnotic effect of
of 1 mg/ml and above by the chimney
to climb up the glass tube within 30 sec
depressant influence of the drug.

Results

1 *Development of tolerance.*

Phenobarbital. The results of our experiments with phenobarbital are summarized in table 1 It can be seen from the table that during a fortnight the drug concentration in the drinking water had to be raised from 0 19 to 1 1 mg/ml, the daily drug intake from 30 to 100 mg/kg in order to maintain 50 / protection against electroconvulsion In parallel the serum concentration, determined for 50 % protection, rose at first rather rapidly from 12 to 22 µg/ml within 4 days and then more slowly to 30 µg/ml after 14 days of treatment. The difference from the value of the first day became significant for all three after 4 days of treatment.

Diphenylhydantoin As can be seen from the results summarized in table 2, the drug concentration in the drinking water providing 50 / protection against electroconvulsion remained constant for the first two days of treatment, but then rose fairly steeply so that after 14 days the concentration had to be increased 7 fold over that of the first two days. Since the mice refused to drink much of the more concentrated solutions (see below), the rise in the daily intake was distinctly smaller amounting to only 2.5-times the DD50 of the first day The serum concentration at 50 % protection increased steadily with the duration of treatment, from 11 to 47 µg/mL

Ethosuccinimide With this compound it proved impossible to construct dose-response curves, since the maximal daily doses taken up – though

Table 1

Development of tolerance to the anticonvulsant effect of phenobarbital.
DC50 = drug concentration in drinking water } providing 50 % protection in the maximal electroshock seizure test
DD50 = average daily drug intake } providing 50 % protection in the maximal electroshock seizure test
SC50 = serum phenobarbital concentration at 50 % protection in this test after different periods of treatment.
The p-values give the significance of the difference from the 1 day-value.

	Period of treatment (days)				
	1	2	4	7	14
DC50 (mg/ml)	0.19 (0.15–0 25)	0.22 (0 18–0.27)	0.44 (0.36–0.54) $p < .01$	0.69 (0.6–0.79) $p < .001$	1 1 (0.94–1.25) $p < .001$
DD50 (mg/kg)	30 (23–38)	32 (22–46)	50 (45–55) $p < .02$	75 (69–82) $p < .001$	100 (94–110) $p < .001$
SC50 (µg/ml)	12 (8–17)	14 (11 18)	22 (16–29) $p < .05$	20 (15–27) $p < .05$	30 (25–.6) $p < .01$

Table 2

Development of tolerance to the anticonvulsant effect of diphenylhydantoin.
DC50 – drug concentration in drinking water } providing 50% protection in the maximal electroshock seizure test
DD50 – average daily drug intake }
SC50 – serum diphenylhydantoin concentration at 50% protection in this test after different periods of treatment.
The p-values give the significance of the difference from the 1 day-value.

	Period of treatment (days)				
	1	2	4	7	14
DC50 (mg/ml)	0.22 (0.16–0.29)	0.21 (0.17–0.27)	0.27 (0.22–0.34)	0.43 (0.34–0.54) $p < .01$	1.4 (1.[illegible] 1.8) $p < .001$
DD50 (mg/kg)	24 (20–29)	23 (20–27)	27 (24–31)	31 (29–34) $p < .05$	59 (56–63) $p < .001$
SC50 (µg/ml)	11 (9–13)	13 (11 16)	18 (13–25) $p < .05$	4 (22–26) $p < .001$	47 (38–58) $p < .001$

up to 1 g/kg – were insufficient to provide more than 20–40 / protection in the pentetrazole seizure threshold test. So we decided to follow the concentrations of 8 and 10 mg/ml drug in the drinking water from which the average daily intake over the different times of treatment amounted to between 500 and 900 mg/kg. Though the daily intake remained fairly constant in all experiments, the anticonvulsant effect showed considerable variation (table 3) and this applied even more to the serum concentrations determined The variation may be explained by the short half-life of ethosuccinimide in the mouse (see below), making it nearly impossible to maintain a constant serum level.

Table 3

Anticonvulsant effect of two concentrations of ethosuccinimide in the drinking water after different periods of treatment. The anticonvulsant effect is determined by the pentetrazole seizure threshold test.

Drug concentration (mg/ml)	Per cent protection after				
	1	2	4	7	14
	days of treatment				
8	35	25	15	15	20
10	40	5	35	5	32

2. *Development of tolerance to the sedative-hypnotic effect of phenobarbital.*

In the phenobarbital experiments running over 14 days, the ability of the mice to climb up backwards in a glass-tube, as described by BOISSIER *et al.* (1960) was tested on 5 days of the week. The results of the test with different concentrations of the drug in the drinking water are shown in fig. 1. Within the first 2–3 days of treatment the ability of 30–60 % of the mice to climb up in the tube was impaired. This primary depression was followed by nearly full recovery and a secondary depression, most pronounced at the highest concentration. Since in the second week of treatment some deaths occurred at all concentrations used, this secondary depression can be looked upon as a consequence of a severe chronic barbiturate intoxication breaking through the state of tolerance previously acquired.

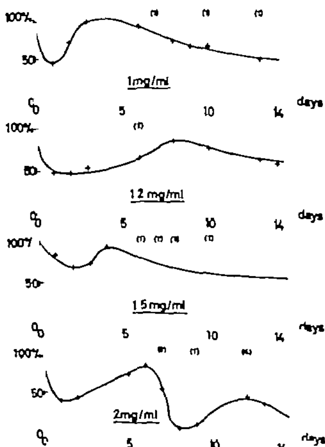


Fig. 1. Performance of mice in the chimney test of BOISSIER *et al.* (1960) under the influence of various concentrations of phenobarbital in the drinking water. The ordinate gives the percentage of animals able to climb up the tube within 30 sec. The small figures in parentheses mark the deaths occurring under the treatment.

3 *Toxic manifestations of treatment*

There was some loss of weight in the groups receiving the higher drug concentrations (table 4), especially in the experiments of longer duration. These losses were most pronounced with diphenylhydantoin, but this can probably be explained by the strongly reduced fluid intake in those experiments in which its concentration was above 0.3 mg/ml (table 5).

Deaths occurred only in the 14 days experiments, beginning at a concentration of 1 mg/ml phenobarbital and 2 mg/ml diphenylhydantoin (table 6)

In the experiments with 8 mg/ml ethosuccinimide in the drinking water running over 7 and 14 days, the mice lost about 10 / of their initial weight at a concentration of 10 mg/ml the weight loss amounted to about

Table 4

Drug concentrations in the drinking water causing weight losses exceeding 10 % during the experiment.

Duration of treatment d ys	Concentration (mg/ml) causing more than 10 % weight loss	
	Phenobarbital	Diphenyl-hydantoin
4	>0.6¹)	>0.4¹)
7	>1)	from 0.5
14	from 1	from 1

¹) H ghest concentration tested.

Table 5

Drug concentrations (mg/ml) in the drinking water leading to reduced fluid intake.

Duration of treatment (days)	Concentration causing a reduction of fluid intake to below		
	3 ml/mouse/day phenobarbital	2 ml/mouse/day phenobarbital	2 ml/mouse/day diphenylhydantoin
1	>0.5¹)	>0.5¹)	f om 0 4
	>0 4¹)	>0.4¹)	from 0.4
4	from 0 6	from 0.6	from 0 4
7	from 1	from 0.5	from 0.3
14	from 1	<1²)	<0.7²)

¹) Highest concentration tested. ²) Lowest concentration tested.

Table 6

Deaths occurring under treatment with higher concentrations of phenobarbital and diphenylhydantoin in the drinking water

Duration of treatment (days)	Phenobarbital		Diphenylhydantoin	
	concentration (mg/ml)	deaths	concentration (mg/ml)	deaths
7	up to 1	none	up to 1.5	none
14	1	3/20	up to 1.5	none
	1.2	2/40	2	3/20
	1.5	4/20		
	2	22/40		

10 / after 7 days and 13–16 / after 14 days. In the 14 days experiment at the higher concentration, one out of 20 mice died. With both concentrations the daily fluid intake lay below 2 ml/mouse.

4 *Sex differences*

Sex differences to the anticonvulsant effect of the different drug concentrations in the drinking water were not very pronounced. However with phenobarbital the female groups showed protection lying on the average 7.5 to 23 / above the corresponding male groups in all experiments lasting for 2 days or longer In the experiments with diphenylhydantoin there was a slight trend to better protection of the male mice in the short experiments (1 and 2 days), whereas in the 14 days experiments the females on the average proved to be 10 / more protected than the males.

5. *Half-life time*

The half life was found in mice of both sexes after intravenous injection to be 6.5 hours for phenobarbital, 33 hours for diphenylhydantoin and 1 hour for ethosuccinimide.

Discussion

Our results with phenobarbital and diphenylhydantoin clearly show that tolerance to the anticonvulsant action of these drugs develops in a short time. For phenobarbital some temporal coincidence with the development of tolerance to the sedative effect of the drug could be shown, suggesting that the tolerance does not differentiate between the different effects of the drug, as was assumed by HENRIKSEN & KRISTIANSEN (1964).

The extent of the tolerance was considerable, as can be seen by comparing the necessary drug concentrations and doses and the toxic manifestations of the administration (tables 1 2, 4–6) With phenobarbital the drug concentration in the drinking water necessary to produce protection against electroconvulsion gave rise to some reduction in water intake already after 7 days of treatment after 14 days the mice had reached a state of chronic barbiturate intoxication, as is shown by the loss of acquired tolerance to the sedative-hypnotic effect (fig. 1), appreciable loss of weight and the incidence of some deaths at all concentrations in the drinking water above 1 mg/ml.

The toxic manifestations were still more pronounced in the experiments with diphenylhydantoin, but judgement is here complicated by the fact that the mice refused to drink the rather alkaline drug solution. Thus the water intake per mouse per day lay below 3 ml in all experiments, and weight losses occurred beginning with a drug concentration of 0.5 mg/ml This concentration lies slightly above the DC50 for 7 days treatment Likewise all drug concentrations tested for determining the protective effect after 14 days of treatment gave rise to serious reduction in water intake and weight loss, though deaths were observed only at the highest concentration used, i e 2 mg/ml.

According to our present knowledge the phenomenon of tolerance to drug action can be explained by two different and independent mechanisms. The one of these is a central adaptation to the permanent presence of drug, resulting in an enhanced drug concentration necessary to display the same measurable drug effect. In our experiments this factor appears as a raised serum concentration necessary to provide 50 / protection in the maximal electroshock procedure (SC50) The other factor is an acceleration of drug metabolism by induction of enzymes located in the microsomes of the liver This factor which has been investigated extensively by REMMER and coworkers (vide REMMER 1962d) in recent years, should in our experiments have been demonstrable by a rise in the daily dose necessary (DD50) to something exceeding that of the SC50 Comparison of the relative rise in DD50 and SC50 during the time of our experiments would thus permit estimation of the role the two factors mentioned play in the development of drug tolerance In fig. 2 the theoretical possibilities are depicted schematically In case a) both values remain at the initial level no tolerance occurs. In b) only the DD50 rises, the SC50 remaining at the initial level here an accelerated drug metabolism alone is responsible for the developing tolerance. In c) the rise in DD50 exceeds that in SC50, central adaptation and acceleration of drug metabolism both playing part in the tolerance. In example d) both factors rise to the same degree here the tolerance is brought about by central adaptation

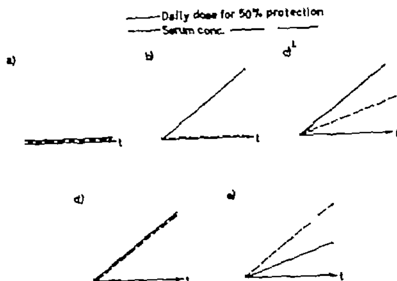


Fig. 2. Schematic presentation of the possibilities of relative rise (ordinate) in daily drug intak (————) necessary to provide 50% effect and the serum concentration (- - - - -) at 50% effect under the development of tolerance. The abscissa gives the time. a) no tolerance. b) tolerance from accelerated drug metabolism. – c) tolerance from both central adaptation and accelerated drug metabolism. d) tolerance from central adaptation only e) tolerance from central adaptation t an accumulating drug. For further explanation, see text.

alone, the rise in drug intake being necessary to provide the higher serum concentration. The special case of e) were the rise of SC50 exceeds that of DD50 can be explained by central adaptation to an accumulating compound.

In this investigation (fig. 3) we see that the DD50 and the SC50 of phenobarbital show an equal rise during the first 4 days of treatment, thus pointing to a developing central adaptation to the drug in this period. Then the rise in the SC50 becomes slower and is exceeded by the steadily rising daily intake. It can therefore be concluded that in the last two thirds of the experimental period an acceleration in drug metabolism by enzymatic induction becomes involved besides the central adaptation already existent and now only proceeding slowly. With diphenylhydantoin we find present the special condition e) of our schematic presentation in fig. 2, i.e. the SC50 shows a greater rise than the daily intake. This points to a predominant role of central adaptation to a drug accumulating in the organism. A minor role of accelerated drug metabolism, however cannot be excluded with certainty. The accumulation of diphenylhydantoin in the mouse is explained by the half-life of 33 hours in this species. Thus the mouse behaves in just the opposite way from man in the elimination of phenobarbital and diphenylhydantoin. In man phenobarbital

Summary

In experiments on mice it could be shown that a tolerance to the anticonvulsant effect of phenobarbital and diphenylhydantoin develops within a few days. After 14 days of treatment the daily dose of phenobarbital providing 50 % protection in the maximal electroshock seizure test lay more than 3 times above the initial level, and the corresponding serum concentration had risen from 12 to 30 µg/ml. In the experiments with diphenylhydantoin the daily dose had to be increased 2.5-fold and the serum concentration at 50 % protection rose more than 4-fold within 14 days. A comparison of the relative rise in serum concentration and daily intake showed that the tolerance to phenobarbital was brought about by central adaptation and accelerated enzymatic oxidation of the drug, whereas the tolerance to diphenylhydantoin was due to a central adaptation to the accumulating compound.

In experiments with ethosuccinimide the mice did not take up enough drug with the drinking water to provide full protection in the pentetrazole seizure threshold test. Results with two partly effective concentrations gave no clear evidence for the development of tolerance to the drug, which is metabolized extremely rapid in the mouse.

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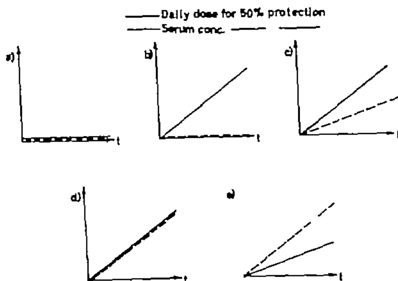


Fig. 2. Schematic presentation of the possibilities of relative rise (ordinate) in daily drug intake (————) necessary to provide 50 % effect and the serum concentration (— — —) at 50 % effect under the development of tolerance. The abscissa gives the time. a) no tolerance. b) tolerance from accelerated drug metabolism. – c) tolerance from both central adaptation and accelerated drug metabolism. d) tolerance from central adaptation only – e) tolerance from central adaptation to an accumulating drug. For further explanation, see text.

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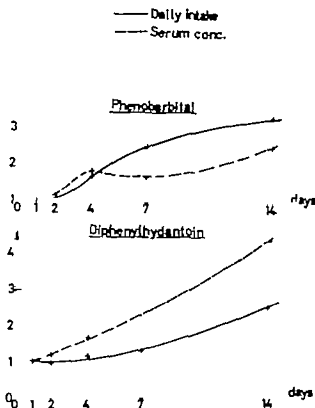


Fig. 3. Relative rise of daily drug intake necessary to provide 50% protection in the maximal electroshock seizure test (————) and of the serum concentration at 50% protection (– – – – –) under the development of tolerance to the anticonvulsant effect of phenobarbital and diphenylhydantoin.

has a half life of about 3 days (SUNSHINE & HACKETT 1959) as opposed to about 24 hours for diphenylhydantoin (BUTLER 1957)

The question of how far results obtained on the mouse permit us to draw conclusions for clinical application to man is difficult to answer Considering the fact that the metabolic activity of mice in oxidizing drugs may be 20 to 60 times higher than that of man (BRODIE *et al.* 1958), one may look upon the mouse experiment as an accelerated photograph of the conditions in man. Transferred to our present investigation, this would mean that the process of developing tolerance to anticonvulsant effects – being a matter of days in the mouse – may take months or even years in man. The clinical observation of JOVO (1964), according to which anti-epileptics as a whole show a tendency to reduced effectiveness already after 2 to 4 weeks of treatment, can on the other hand be interpreted as an indication that the gap between mouse and man might

not be over large for phenobarbital and diphenylhydantoin, of which the former is metabolized rather slowly also by the mouse, whereas for the latter the half-life time in both species is practically identical. The fact that most clinical workers have not noted the existence of tolerance to anti-epileptic medication may be explained in several ways. 1 The tole rance may already have been established while the effective anticonvulsant dose is still being adjusted to the patient s special requirements. 2. The accumulation occurring under the treatment may especially with phenobarbital, obscure the development of tolerance, since it provides serum and tissue concentrations that, even during a state of tolerance, may be sufficient to suppress the convulsive disorder The serum concentrations found by Lous (1954) and in our own unpublished investigations in patients under chronic treatment with phenobarbital lie, in fact, partly above the SC50 we have found in mice after 14 days of treatment, i.e. at a time when tolerance is firmly established. 3 The anticonvulsant effect in the patient cannot be stated with the numerical exactness of that based on animal experimentation, since many external factors may interfere with the patient s special clinical state.

In our experiments with ethosuccinimide, even a daily intake of 0 8 to 1 g/kg was not sufficient to provide more than 20-40 / protection against the chemoconvulsion provoked by subcutaneous injection of pentetrazole, and higher doses were not taken up by the animals. A follow-up study of two partly effective concentrations showed a relatively high variation in anticonvulsant effect, but no tendency towards a reduced effectiveness after the longer periods of treatment. The same rather high variation was seen in the serum concentration of the drug and can best be explained by the short half-life of ethosuccinimide in the mouse. Thus, although we got no positive evidence for the development of tolerance to ethosucci nimide in this species, it seems not to be permissible to draw from them conclusions applicable to man in whom the much longer half-life (Hansen & Feldberg 1964) might provide entirely different conditions.

A comparison of our results with those on some anticonvulsant drugs reveals some parallelism with results of Remmer and coworkers (1960, 1962a & c) obtained for different barbituric acid derivatives. These authors, also reached the conclusion that tolerance develops more easily to the slowly metabolized compounds, as for example phenobarbital, than to barbiturates oxidized rapidly in the body Whereas for phenobarbital both central adaptation and acceleration of drug metabolism contribute to the development of tolerance, under chronic treatment with readily oxidizable barbiturates only an increased metabolism (N-methylpropallylonal, butallylonal) or no tolerance at all (pentobarbital, thiopental) could be demonstrated in the mouse.

Summary

In experiments on mice it could be shown that a tolerance to the anticonvulsant effect of phenobarbital and diphenylhydantoin develops within a few days. After 14 days of treatment the daily dose of phenobarbital providing 50 / protection in the maximal electroshock seizure test lay more than 3 times above the initial level, and the corresponding serum concentration had risen from 12 to 30 µg/ml. In the experiments with diphenylhydantoin the daily dose had to be increased 2.5-fold and the serum concentration at 50 / protection rose more than 4-fold within 14 days A comparison of the relative rise in serum concentration and daily intake showed that the tolerance to phenobarbital was brought about by central adaptation and accelerated enzymatic oxidation of the drug, whereas the tolerance to diphenylhydantoin was due to a central adaptation to the accumulating compound.

In experiments with ethosuccinimide the mice did not take up enough drug with the drinking water to provide full protection in the pentetrazole seizure threshold test. Results with two partly effective concentrations gave no clear evidence for the development of tolerance to the drug, which is metabolized extremely rapid in the mouse.

Acknowledgements

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Effect of p-Hydroxyamphetamine on Catecholamine Excretion in Man

By

Wilfried von Studnitz

(Received October 14 1964)

In 1962 CREVELING DALY WITKOP & UDENFRIEND and GOLDSTEIN & CONTRERA showed that *p*-hydroxyamphetamine is a substrate for the so-called dopamine-β-oxidase *in vitro* Our investigations indicate that this is also true *in vivo* (SJOERDSMA & V STUDNITZ 1963) CHIDSEY HARRISON & BRAUNWALD (1962) were able to demonstrate a release of noradrenaline from dog heart by *p*-hydroxyamphetamine.

The purpose of the study reported here was to investigate any possible effect of *p*-hydroxyamphetamine on catecholamine metabolism in man.

Materials and Method

Five hospitalized patients without any significant liver disease were studied. Three of them had mild hypertension, and two were normotensive. All received a constant diet during the study 20 mg of *p*-hydroxyamphetamine (as hydrobromide) were administered orally every 6 hours for 4 to 6 days.

Twentyfour hour urine specimens were collected in glass bottles containing 15 ml 6 N HCl during 3 control days, during the period of drug administration and 2 days after treatment.

The 3-methoxy-4-hydroxymandelic acid (VMA) was measured by the method of PISANO CROUT & ABRAHAM (1961). Urinary noradrenaline (NA) was assayed fluorometrically by the trihydroxyindole procedure involving ferricyanide oxidation at pH 6, as described by CARLSSON *et al.* (1959)

In all patients except one (G.I.) no side effects from the drug and no effects on blood pressure were noted. Patient G I showed some increase in her systolic and diastolic blood pressure. This patient received additional steroids equivalent to 50 mg cortisone for other reasons during the second half of the drug period.

In vitro studies were made of the inhibitory effect of *p*-hydroxyamphetamine on the formation of noradrenaline from dopamine. Enzyme preparations of the kind described by LEVIN *et al.* (1960) were used. The reaction mixtures contained the compo-

ments (in micromoles) phosphate buffer pH 6.., 100 fumaric acid, 10 ascorbic acid 10 catalase (saturated aqueous solution of crystalline bovine catalase) 20 µl dopamine, 0.1 5 p-hydroxyamphetamine, 2. The final volume of the reaction mixture was 1 ml Incubation time at 36° 20 minutes. The reaction was stopped by adding 3 / trichloroacetic acid. Noradrenaline was assayed by the trihydroxyindole method of v Euler *et al.* (1955) adapted to the Aminco-Bowman spectrophotofluorometer

Results

The urinary VMA showed a tendency to decrease during the administration of *p*-hydroxyamphetamine in all five patients. The observed decrease was not striking, and no value fell below 50 / of the control values. The results are shown in figs. 1 to 3 In the same figures the falls in urinary NA excretion in 4 out of 5 patients are also shown. After withdrawal of the drug, both VMA and NA began to rise again and returned almost to control levels. Patient G I. who also received steroids, showed no difference in response to *p*-hydroxyamphetamine from the other patients.

Our *in vitro* studies with dopamine-β-oxidase showed that *p*-hydroxyamphetamine inhibits the formation of noradrenaline from dopamine. The inhibition is of the competitive type (see fig. 4). The Michaelis constant (K_m) for p-hydroxyamphetamine was $1\,5 \times 10^{3}$ moles per litre.

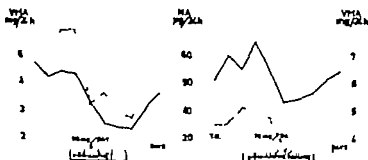


Fig. 1 The urinary excretion of noradrenaline (———) and 3-methoxy-4-hydroxymandelic acid (———) before, during and after administration of *p*-hydroxyamphetamine. See text.



Fig. .. See text to fig. 1

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In vitro studies were made of the inhibitory effect of *p*-hydroxyamphetamine on the formation of noradrenaline from dopamine Enzyme preparations of the kind described by Levin *et al.* (1960) were used. The reaction mixtures contained the compo-

type. With such a degree of inhibition *in vitro* one would normally not expect any significant inhibition *in vivo* by the dose employed in our studies. However *in vitro* results cannot always be applied to *in vivo* conditions.

The presence of dopamine-β-oxidase activity in the adrenal medulla, brain stem areas (UDENFRIEND & CREVELING 1959) and the adrenergic nerves (CARLSSON & WALDECK 1963) of various laboratory animals is well established. Little, however is known about the actions of this enzyme in the human body and at the present time nothing about the distribution of *p*-hydroxyamphetamine in the body The amount of β-hydroxylated compounds from endogenous sources excreted under normal conditions is less than 10 mg/24 hour urine volume. They consist of the methoxy catecholamines, the VMA, dihydroxymandelic acid, noradrenaline, adrenaline and other known catecholamine metabolites. The patients in our study received up to 12 times more compound of which at least 50 / is absorbed (SJOERDSMA & v STUDNITZ 1963) after oral administration. The results of our study are consistent with an inhibition of dopamine-β-oxidase by *p*-hydroxyamphetamine. On the other hand one must also bear in mind that administration of a transmittor analoque, such as *p*-hydroxyamphetamine, could lead to a decrease in activity of the sympathic system. Further experimental studies to clarify this problem are in progress.

Summary

A decrease in urinary excretion of noradrenaline and 3-methoxy-4-hydroxymandelic acid in man could be observed after oral administration of *p*-hydroxyamphetamine. *In vitro* studies showed that this amine inhibits the formation of noradrenaline from dopamine. The possible relationships between these findings are discussed.

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Excretion of Human Urinary Kinins

By

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(Received September 2, 1964)

Methods have been described for the purification of the kinins of human urine (BRISEID JENSEN 1958 BRISEID JENSEN & SUND 1959) and also for their separation into two main fractions, Z_1 and Z_2 (BRISEID JENSEN & VENNERÖD 1962a & c BRISEID JENSEN, RINVIK & VENNERÖD 1963). Substance Z_1 could not be distinguished from bradykinin in the chromatographic or pharmacological tests used, whereas the Z_2 fraction was closely similar to kallidin (BRISEID JENSEN & VENNERÖD 1962b & c BRISEID JENSEN, VENNERÖD & DYRUD 1963 BRISEID JENSEN, RINVIK & VENNERÖD 1963).

HORTON (1959) estimated the total kinin excretion in rather crude urine specimens. He attempted to establish the specificity of his method by the combined use of rat uterus and rat duodenum for the assays. He pointed out, however that wasp kinin could not be distinguished from the plasma kinins by parallel assays on those muscles and concluded that the method could probably not distinguish between closely allied but chemically different kinins. As previously mentioned (BRISEID JENSEN & VENNERÖD 1962c), the procedure did not distinguish between the urinary kinin fractions Z_1 and Z_2

In our work we have adapted the purification and separation methods previously described by us for determining the excretion of both kinin fractions in 24-hour urine specimens. Its main purpose was to estimate the excretion of Z_1 and Z_2 in a sufficiently large number of healthy males to give an idea of the normal excretion values. We have also carried out some determinations of the kinins in urine from subjects suffering from different diseases.

Technique

A. Materials and assay

I Urine Specimens were collected over 24 hours in polythene bottles containing 100 ml 1.3 N hydrochloric acid to inactivate the kininase present. The pH was adjusted to about 2 when necessary (with N-hydrochloric acid or N-sodium hydroxide), and the urine was kept at −17° until required. Usually 3 urine specimens were collected from each subject at intervals over a period of about one month.

II Standard preparation. Both the Z_1 and the Z_2 concentrates were assayed on rat uterus against synthetic bradykinin (Sandoz A.G., Basel, Switzerland). The results of the Z_1 assays were given as μg bradykinin, but the Z_2 values were calculated as kalfidin, which has only about 60 % of the activity of bradykinin on rat uterus (STÜRMER & BERDE 1963)

III Assays The active fractions were assayed on the isolated rat uterus by comparison of the unknown with two doses of standard, usually in the ratio 3 : 4.

B Preparation of concentrates of Z_1 and Z_2

The technique used for preparing concentrates of the two kinin fractions has been previously described and discussed by BRISEID JENSEN & SUND (1959), BRISEID JENSEN & VENNERÖD (1962) and BRISEID JENSEN, RINVIK & VENNERÖD (1963). However in order that the procedure may be more easily surveyed, the different steps will be described below and for the volumes of urine used.

I Adsorption of activity A volume of the urine and hydrochloric acid mixture corresponding to 1000 ml 24-hour urine (or the total 24-hour urine, when less than 1000 ml was collected) was adjusted to pH 6.0 ± 0.1 with 1 N sodium hydroxide. To this were added 1 litre of water and about 4.5 g of Amberlite CG-50 (100–200 mesh) pretreated and buffered to pH 6.0 ± 0.1 as previously described (BRISEID JENSEN & SUND 1959). The mixture was stirred vigorously for about 30 minutes.

II Washing The resin was allowed to settle most of the fluid could be decanted and discarded. The rest of the fluid was sucked through a 6.5 cm diameter sintered filter of porosity 3 (Baird & Tatloch) connected to a water pump. The resin was then stirred in 100 ml of distilled water for a few minutes, and the water was sucked off. This procedure was repeated three times. After washing with 1 N acetic acid, 4 × 60 ml portions, the mixture being stirred each time for about 15 minutes, the resin was washed with distilled water to remove the acetic acid, 7 × 60 ml portions of water being used.

Finally the resin was stirred for about 30 minutes with 110 ml of 0.3 N sodium hydroxide (7.8 meq per g resin), resulting in a pH of 9.2–10.0 in the supernatant. The slightly yellowish fluid was practically inactive and was sucked off and discarded. The residue was then washed with about 110 ml of distilled water which was also sucked off and discarded.

III Elution of the kinins. The pH of the supernatant fluid was brought to 12–13 by stirring for half an hour with 45 ml of 0.3 N sodium hydroxide (3 meq per g resin). The fluid, containing nearly all the activity was sucked off and concentrated hydrochloric acid was added to give a pH of about 1.5 ± 0.1

Two samples of 1 ml each were taken for the estimation of total kinin present

($Z_1 + Z_2$). They were evaporated in round-bottomed flasks under reduced pressure (5–10 mm Hg) on a rotating evaporator and kept over silica gel in evaporated desiccators at room temperature for later assay (*a*).

The rest of the active eluate was saturated with sodium chloride (40 g/100 ml fluid) and shaken twice with the same volume of freshly distilled n-butanol, each time removing all Z_1 and about $\frac{1}{3}$ of the Z_2 present. The transferred activity was extracted from the butanol by shaking three times with distilled water the volume of water each time corresponding to $\frac{1}{3}$ of the butanol volume. Two samples of 2 ml each were taken for the estimation of $Z_1 + \frac{1}{3} Z_2$ (*b*).

In the same way 2 samples of 2 ml each were taken from the remainder of the saturated sodium chloride solution for the estimation of $\frac{2}{3} Z_2$ (*c*). The *b* and *c* samples for assay were evaporated and kept as described above for the $Z_1 + Z_2$ samples (*a*). The kinin contents of all samples were determined as μg bradykinin and the figures adjusted to the original amount of 24-hour urine before calculation.

I) Calculations To the figure obtained for c was added $\frac{1}{2}c$ giving the total amount of Z_2 in terms of bradykinin. On dividing by 0.6 the result was transferred to μg kallidin.

By subtracting $\frac{1}{2}$ *c* from *b* we obtained the amount of Z_1 present in terms of bradykinin.

The value for total kinin present (*a*) was used to check the loss of activity during the separation procedure. From a previous publication (BARSEBO JENSEN & VINOSVALDO 1963) it can be seen that some loss usually occurs. In the work reported here the mean recovery of kinin fraction Z_1 + kinin fraction Z_2 (urines from 12 healthy males, table I) was 82% ± 6.5 (*s*).

Results

A. *Normal urine*

The excretion of the kinin fractions Z_1 and Z_2 was estimated in 12 healthy males, all within the age range *of 25 to 55* years. The results are shown in table 1 The amounts of kinins excreted are given as μg substance per 24 hours and calculated as bradykinin for Z_1 and kallidin for Z_2 Urine volumes in ml are also given in the table. It can be seen that the daily output of kinins was not related to the volume of urine. HORTON (1959) has previously found the same in experiments in which the rate of urine formation was adjusted by varying the fluid intake.

Table 1 further shows that the average values of Z_1 and of Z_2 for the 12 persons were roughly the same, $Z_1/Z_2 = 0.8$. Also the average values of Z_1 and Z_2 for the 3 urine specimens from each subject were of the same order of size, Z_1/Z_2 ranging from 0 6 to 1 1 This means that, if the present material can be considered representative for normal male urines, a determination of total urinary kinin should also give approximate values for the amounts of the two kinin fractions present. The variations between the individual urine specimens from each person, however were wide enough to indicate that such a determination should be based on the combined results from at least 3 24-hour urine specimens.

Table I

Urinary excretion of kinin fractions Z_1 and Z_2 in healthy males.

The kinin values refer to 24-hour urines.
The Z_1 values are expressed as μg bradykinin, the Z_2 values as μg kallidin.
The fractions were tested on rat uterus.

Subject number	ml urine	Z_1		Z_2		$Z_1 + Z_2$
1	1290	7.0 4.5 8.0	6.5	1[illegible].5 7.8 10.0	10.1	16.6
2	1560	7.5 9.9 4.5	7.3	8.5 18.3 9.5	12.1	19.4
3	1540	8.7 23.1 19.4	17.1	30.5 23.5 33.3	29.1	46.2
4	1810	3.7 19.9 20.7	14.8	26.3 17.5 12.5	18.8	33.6
5	1100	19.4 13.2 8.9	13.8	18.0 9.0 9.3	12.1	25.9
6	1570	12.9 7.0 20.5	13.5	19.0 14.0 23.0	18.7	32.2
7	1270	14.0 9.6 26.2	16.6	10.3 12.5 22.8	15.2	31.8
8	1500	16.6 16.2 35.7 22.0	22.6	24.3 30.0 38.3 39.8	33.1	55.7
9	1230	16.1 9.4 8.7	11.4	19.5 15.8 16.8	17.4	28.8
10	860	31.1 3.4 22.3	18.9	32.0 20.8 28.0	26.9	45.8
11	1010	17.7 10.3 13.9	14.0	15.8 11.5 13.0	13.4	27.4
12	1400	31.4 18.9 20.9	23.7	29.3 17.8 23.0	23.3	47.0
Averages			15.0		19.2	34.2 (± 12)

Table 1 shows that the mean excretion value of $Z_1 + Z_2$ for the 12 subjects was 34.2 µg with a standard deviation of 12.1

From table 1 it can be further seen that the average excretion values of $Z_1 + Z_2$ for each subject ranged from 16.6 to 55 7 µg per 24 hours this gives a theoretical mean value of about 36 µg and maximum deviations of somewhat more than 50 %. The absolute quantities are difficult to compare with the data of HORTON (1959), who based his values on a urinary kinin standard preparation instead of on bradykinin and kallidin. The range of means of excretion values in Horton's experiments varied from 6.5 to 17.5 units/min., which gives a mean of 12 units and maximum deviations of somewhat less than 50 %.

Table 2

Urinary excretion of kinin fractions Z_1 and Z_2 in pregnant women.

The kinin values refer to 24-hour urines collected during the last two months of pregnancy The Z_1 values are expressed as µg bradykinin, the Z_2 values as µg kallidin. The fractions were tested on rat uterus.

Subject number	Z_1		Z_2		$Z_1 + Z_2$
1	13.0 9.6 15 7	12.8	22.3 15.5 22.8	20.2	33.0
2	50 3 25 7 31 3	35.8	32.5 22.5 28.0	27 7	63.5
3	13.0 21.8 19 8	18.2	18.3 23.8 25.8	22.6	40.8
4	20.5 21 4 11 5	17.8	19.3 12.5 10.0	13.9	31 7
5	8.7 8.9 11.2	9.6	8.0 7.8 8.3	8.0	17 6
6	49 9 31.5 50.6	44.0	44.0 37.3 44 8	42.0	86.0
7		25.5		19.3	44.8
8		35.2		26.8	62.0
9		29.3		15.0	44.3
10		28.1		29.3	57 4
Averages		25 6		22.5	48.1

B Urine from pregnant women

The urinary excretion of Z_1 and Z_2 was tested in pregnant women, 10 of whom were considered healthy while 5 others were hospitalized for pregnancy toxaemia. Three 24-hour urine specimens were collected during the two last months of pregnancy but some of the women gave birth before all the specimens could be collected. The results are shown in tables 2 and 3

It can be seen that the average values for total kinin excretion ($Z_1 + Z_2$) in the healthy subjects (table 2) was higher than that for the normal material from males (48.1 µg as against 34.2 µg) The increase in kinin excretion was due to a higher output of Z_1 the Z_2 figure being nearly the same ($Z_1/Z_2 = 1.1$ as against 0.8)

Table 3 shows that the average total output of kinins in urine from women with pregnancy toxaemia was fairly close to the output in healthy pregnant women (51.6 µg as against 48.1 µg), though the Z_1 fraction made up a significantly larger part of the excretion ($Z_1/Z_2 = 2.0$).

Table 3

Urinary excretion of kinin fractions Z_1 and Z_2 in women with pregnancy toxaemia.

The kinin values refer to 24-hour urines collected during the last two months of pregnancy The Z_1 values are expressed as µg bradykinin, the Z_2 values as µg kallidin.
The fractions were tested on rat uterus.

Subject number	Z_1		Z_2		$Z_1 + Z_2$
1	29.7 41.4 26.3	32.5	13.5 23.8 12.5	16.6	49.1
2	30.3 42.5 46.2	39.7	16.3 22.3 18.8	19.1	58.8
3	33.3 52.4 75.3	53.7	18.5 16.0 22.8	19.1	72.8
4		36.3		27.3	63.6
5		10.8		3.0	13.8
Averages		34.6		17.0	51.6

C. Urine from subjects with different diseases.

Table 4 gives some figures for kinin excretion by subjects suffering from different diseases, eczemas, essential hypertension, rheumatoid

arthritis. The results are based on few observations only and accordingly may not be representative. However the fact that some kinin excretion values clearly deviate from normal values suggests the desirability of examining a larger body of material.

Table 4 shows first the output of kinins in 4 males with eczema. The average values for total excretion were of the same order as those for the normal urine specimens (table 1) and the Z_1/Z_2 ratio was about the same.

Table 4

Urinary excretion of kinin fractions Z_1 and Z_2 in pathological conditions.

The kinin values refer to 24-hour urine specimens.
The Z_1 values are expressed as µg bradykinin, the Z_2 values as µg kallidin.
The fractions were tested on rat uterus.

Subject number	Sex	Diagnosis	Z_1		Z_2		$Z_1 + Z_2$
1	M	Eczema Erythrodermia	18.9 8.3	13.6	12.3 7.0	9 6	23.2
2		Eczema pruriginosum	28.7 35 6	32.2	29.3 32.0	30.7	62.9
3			21.8 31.0	26.4	19.0 33.3	26.2	52.6
4			13.3 11 1	12.2	13.3 12.0	12.7	24.9
Averages				21 1		19.8	40.9
1	F	Essential hypertension	21.2 28.5 26.9	25.5	7.3 11.0 11.8	10.0	35.5
2	M		27 2 13.6 35 9	25 6	11.3 13.3 21.3	15.3	40.9
Averages				25.6		12.7	38.2
1	M	Rheumatoid arthritis	8 6 7.2	7 9	6.7 2.5	4.6	12.5
2	F		7.4 6.6 11.4	8.5	6.5 3 0 7 0	5.5	14.0
Averages				8 2		5.1	13.3

The kinin excretion values for 1 male and 1 female with essential hypertension are also shown in the table. The total output of kinin was normal, but the Z_1/Z_2 value was somewhat high.

The values for excretion by one male and one female with rheumatoid arthritis were low, the average total output of kinins in 2 urine specimens collected at an interval of 4 months and 3 specimens collected over a period of some weeks being lower than for all the normal subjects. Here, too, the Z_1/Z_2 ratio was high, 1.6.

Discussion

The question is not settled whether the urinary kinins are formed locally in the kidney or whether they represent the renal clearance of kinins from plasma. It might well be that both are correct. Small amounts of kinins might be continuously present in plasma as a result of the functional state and the relative effectiveness of releasing mechanisms and inactivating enzymes. On the other hand, it should be remembered that urine kallikrein, like other glandular kallikreins, is clearly distinguishable from plasma kallikrein, thus suggesting some special function in the kidney. The identities of the urinary kinins have considerable relevance to the question of their origin. In a recent publication WEBSTER & PIERCE (1963) show that human plasma kallikrein releases bradykinin (kallidin-9) directly from kallidinogen, whereas human urinary kallikrein gives kallidin (kallidin-10). The kallidin is then rapidly converted to bradykinin by an aminopeptidase present in the plasma. The same authors consider human plasma kallidinogen to be identical with bradykininogen. Though this question is not finally settled, other research workers have also presented evidence for such a view (WERLE, KEHL & KOBKE 1950, HABERMANN 1963). On the other hand, the experiments of VAN ARMAN (1955) were not entirely conclusive. It must be pointed out that the compositions of the urinary kinin fractions Z_1 and Z_2 are not fully established. Previous chromatographic and pharmacological experiments (BRISEID JENSEN & VENNERÖD 1962b & c, BRISEID JENSEN, VENNERÖD & DYRUD 1963, BRISEID JENSEN, RINVIK & VENNERÖD 1963) suggest that bradykinin is the main constituent of Z_1 and kallidin of Z_2, but the Z_2 fraction must contain other active substances. The pharmacological parallel assays, however, show that the unknown substances must either be closely similar to kallidin or present in small quantities only. Accordingly we feel justified in regarding the main constituents of Z_1 and Z_2 as identical with bradykinin and kallidin, respectively. If Z_2 consists mainly of kallidin, it cannot, according to WEBSTER & PIERCE (1963), be released by plasma kallikrein, but only by urinary kallikrein or other glandular kallikreins.

When estimating the excretion results given in this paper it must be borne in mind that the relative amounts of Z_1 and Z_2 found will partly depend on the aminopeptidase activity which is present not only in plasma, but possibly also in urine (WEBSTER & PIERCE 1963). This fact excludes any overemphasis on the significance of the separate determination of the two kinin fractions. The possibility exists, however that under special pathological conditions extremely high or low quantities of one of the kinins might be released, so that an irregular Z_1/Z_2 ratio would be found in spite of the rapid enzymatic conversion process. The somewhat higher ratio observed for women with pregnancy toxaemia than for healthy pregnant women (table 3) might be significant. Accordingly if a similarly decreased excretion of kinins can be established in more subjects with rheumatoid arthritis, as was registered for the single male and the single female (table 4), it might reflect a highly significant decrease in urine kinin level.

Summary

Methods previously devised for the purification and separation of two kinin fractions in human urine, Z_1 and Z_2, have been adapted to their determination in 24-hour urine specimens. Previous chromatographic and pharmacological experiments suggested that bradykinin was the main constituent of Z_1 and kallidin of Z_2. The kinins were assayed on the isolated rat uterus, Z_1 being calculated as µg bradykinin and Z_2 as µg kallidin.

This paper gives the results of determining the two kinin fractions in urines from 12 healthy males and 15 pregnant women, 10 of whom were considered healthy whereas 5 others were hospitalized for pregnancy toxaemia.

Kinin excretion was investigated also in some subjects suffering from different diseases eczemas, essential hypertension, rheumatoid arthritis. The results were based on few observations, but deviated somewhat from the normal, so that further investigation of more material seems advisable.

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Experiments on the In Vitro Inactivation of Plasma Kinins by Carboxypeptidase B, Plasma Kininase or Erythrocyte Kininase in the Presence of Disulfiram

By

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The inhibitory effect of disulfiram (= tetraethylthiuram disulphide) on enzymes catalysing the oxidation of acetaldehyde is generally accepted as the basic mechanism for the effect of the drug on the metabolism of ethyl alcohol. HALD & JACOBSEN (1948), ASMUSSEN HALD & LARSEN (1948), LARSEN (1948) and HALD JACOBSEN & LARSEN (1949) concluded from their experiments that most of the symptoms produced by intake of alcohol in humans treated with disulfiram might be attributed to an accumulation of acetaldehyde in the organism. However disulfiram inhibits not only liver aldehyde dehydrogenase (KJELDGAARD 1949 GRAHAM 1951), but also several other enzymes. The structure of disulfiram might suggest a special affinity for divalent metals and consequently an influence on metalloenzymes. Among other complexing agents capable of combining with zinc VALLE (1960) found disulfiram effective as an inhibitor of the alcohol dehydrogenase of yeast. A zinc metalloenzyme in human plasma that might seem of interest is the kininase inactivating kallidin and bradykinin. The enzyme was found to be similar to the carboxypeptidase B of pancreas by ERDÖS & SLOANE (1962) and ERDÖS & WOHLER (1963), who named it carboxypeptidase N

In the work presented here, experiments were carried out with plasma samples prepared in different ways to establish whether an inhibition of plasma kininase by disulfiram could be detected. Plasma samples from human blood and rat blood and also fresh rat blood samples were used. Plasma kinins were released by incubation with padutin ® (hog pancreas kallikrein), human saliva or trypsin. Some experiments with synthetic bradykinin and carboxypeptidase B or erythrocyte kininase in salt solutions were also performed.

Technique

A. Materials

1. *Stable plasma substrate from human blood* was prepared by the method of AMUNDSEN, NUSTAD & WAALER (1963).

2. "*Stable" plasma substrate from human blood with kininase* was prepared in the same way as the stable plasma (1), but the acidification to pH 2 was omitted.

3. *Stable plasma substrate from rat blood* was prepared in the same way as the corresponding substrate from human blood.

4. "*Stable" plasma substrate from rat blood, with kininase* was prepared in the same way as the corresponding substrate from human blood.

5. *Fresh blood* was obtained from rats under light ether anesthesia. The blood was drawn from the vena cava inferior into siliconized syringes containing sodium citrate and kept for not more than two hours at 4° before use.

6. *Erythrocyte kininase preparations.* The erythrocyte fractions from the preparations of human and rat plasma substrates were washed 4 times with 0.9 % sodium chloride solution. The fluid was each time pipetted off after centrifugation at 2200 r.p.m. The washed cells were kept at −20° and haemolysed before the experiments by addition of an equal volume of water and incubation for 5 minutes at 37°. The method of preparation does not completely exclude the presence of leukocytes and thrombocytes.

7. *Human saliva as source of kinin releasing enzymes.* Saliva was diluted 1 + 9 with 0.9 % sodium chloride and centrifuged for 20 minutes at about 2200 r.p.m., as described by AMUNDSEN, NUSTAD & WAALER (1963). The pH was then lowered to 2 by adding 2 N hydrochloric acid, to inactivate the kininase. After 10 minutes at 37° the pH was adjusted to 7.4 by adding 2N sodium hydroxide, and the preparation was stored at −17°.

B. Chemicals.

Bradykinin (synthetic) in ampoules of 100 μg/ml, Sandoz, A.G., Basel, Switzerland.
Carboxypeptidase B (*CPB*), COB 6064. 107 U/mg. Worthington Biochemical Corp. Freehold, New Jersey, U.S.A.
Padutin ® (hog pancreas kallikrein) in ampoules of 10 Biol. Units, Bayer A.G. Leverkusen, Germany.
Disulfiram. Antabus ® Dumex Chemical Division, Dumex Limited, Copenhagen, Denmark.
Trypsin (2 × crystalline) Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

B. Methods

In the experiments with plasma kininase 0.9 ml portions of plasma substrate or fresh blood was used. The experiments with carboxypeptidase B or erythrocyte kininase were mainly carried out in tris buffer (0.3 M, pH 7.3), and with bradykinin as substrate. Disulfiram was added dissolved in 0.05 ml acetone, and bradykinin, carboxy-

peptidase B (CPB), padutin and trypsin in 0.1 ml 0.9 % sodium chloride solution 0.1 ml of the saliva preparation or 0.1 ml of the human erythrocyte preparation was added without further dilution. The rat erythrocyte preparation was diluted 1 + 2 with 0.9 % sodium chloride solution, and 0.1 ml was added to the incubation mixture. In the disulfiram experiments acetone was also added to the control solutions incubated in parallel. The incubations were carried out at 37°. Samples for assay on rat uterus were usually taken at 4-minute intervals in the short-term experiments. The assays were conducted somewhat roughly by bracketing the unknown between known standard doses of bradykinin. The observed kinin activities were calculated as μg bradykinin. As a limit of dilution, 1.0 ml plasma incubation mixture in 100 ml de Jalon's solution was chosen. Taking into consideration the concentrations of bradykinin usually active on rat uterus, this means that about 0.02 μg kinin/ml incubation mixture could be detected.

Results

Effect of disulfiram on the inactivation of bradykinin by carboxypeptidase B (CPB).

Table 1 shows that CPB was less active at pH 5.8 than at pH 7.3, 20 times higher enzyme concentrations being required at the lower pH value. It can further be seen that disulfiram under the chosen conditions strongly inhibited the inactivation of bradykinin at pH 5.8, but had no significant effect at the pH value of blood.

Inactivation by carboxypeptidase B or plasma kininase of the kinins released in human plasma specimens in the presence of disulfiram

Table 2 shows results obtained with 3 different human plasma specimens, 2 of which were stable, whereas the third was only partly stable, in as much as it contained kininase. No activity could be detected in any of the specimens when tested directly as described under Methods (<0.02 μg/ml of kinin as bradykinin being present).

Table 1

Effect of disulfiram on the inactivation of bradykinin by carboxypeptidase B. For details see text.

Bradykinin μg/ml	CPB μg/ml	Disulfiram mg/ml	pH	Medium	% activity	after time in minutes
1.2	0.043	0	7.3	Tris buffer	0	10
		1	"		0	12
	0.9	0	5.8	0.9 % NaCl	0	16
		1	"		100	100

Table 2

Inactivation by carboxypeptidase B or plasma kininase of the kinins released in human plasma substrate specimens i the presence of disulfiram.
The figures given refer to about 1 1 ml (1.00–1 15) incubation mixture with 0.9 ml plasma substrate.

Human plasma	Padutin mU	Saliva dilution ml	CPB µg	Disulfiram mg	Maximum activity as bradykinin µg	% activity	after time [illegible] minutes
1 Stable		0.1			0.8	100	70
1 –		0.1	0.02		0.8	0	40
–		0.1			0.4	100	120
2 –	50				0.4	100	120
2 –	40				0.4	100	164
–	40 30 min. 60°				0.4	100	164
2 –	50		0.09		0.3	0	21
2 –	50		0.09	1	0.3	0	20
3 "Stable" with kininase		0.1			0.4	0	70
3 –		0.1		1	0.4	0	70

The table shows that 0 4–0 8 µg of kinin was released per ml incubation mixture and that padutin and human saliva had the same amounts. As padutin might be supposed to contain some kininase (K. A. HALVORSEN, personal communication) an experiment was carried out with untreated padutin and padutin heated at 62° for 30 minutes Table 2 shows that

Table 3

Inactivation by plasma kininase of kinin released in or added to rat plasma substrate specimens in presence of disulfiram
The figures given refer to about 1 1 ml (1.00–1 15) incubation mixture with 0 9 ml plasma substrate.

Rat plasma	Trypsin µg	Padutin mU	Bradykinin µg	CPB µg	Disulfiram mg	Maximum activity as bradykinin µg	activity	after time [illegible] minutes
1 Stable						0 05	100	82
1				0.09		0 05	100	32
1 –				1.5		0.05	100	31
1 –		1000				0.05	100	22
1 –	350					0.3	100	36
1 –	350				1	0.4	100	20
2 "Stable" with kininase	350							
2 –	350				1			
–			1.5			0.8	0	10
2			1.5		1	0.8	0	10

both padutin solutions released the same amount of kinin from batch 2 of the stable plasma substrate and that the activity was as stable for the heated as for the unheated padutin solution. The stability of the kinins released with saliva, which was free of kininase, was also high, confirming that the method of AMUNDSEN, NUSTAD & WAALER (1963) yielded a stable plasma substrate.

The inhibitory effect of disulfiram on the enzymatic inactivation of the kinins was tested both on a stable plasma substrate with CPB added and on a stable plasma substrate with kininase. Table 2 shows that disulfiram did not inhibit the enzymes under the conditions chosen.

Inactivation by plasma kininase of the kinins released in rat plasma specimens in the presence of disulfiram

Table 3 shows that the stable rat plasma, batch 1 had a basic activity on rat uterus corresponding to about 50 ng bradykinin per ml. The activity however was unspecific, CPB having no influence on it. Lysergide (= LSD), in an amount that almost completely blocked the effect of a 5-hydroxytryptamine dose giving submaximal contractions, partly inhibited the substance(s) stimulating rat uterus. Table 3 further shows that padutin did not set free any kinin, whereas trypsin was effective in doing so. Under the conditions chosen disulfiram did not interfere with kinin release.

In the experiments with human stable plasma it had been shown that disulfiram did not inhibit CPB. The inactivation experiments with rat plasma were accordingly restricted to "stable" plasma, with the kininase retained, and to fresh rat blood. Table 3 shows that no significant quantities of kinins could be detected in rat plasma with the kininase remaining when trypsin was added and that an addition of disulfiram to the incubation mixture gave the same result. The plasma specimens were tested at the shortest possible intervals during incubation, as for the human plasma specimens, and the negative results are probably due to large quantities of kininase being present, compared to those in human plasma (see table 2). To estimate the inactivating capacity of rat plasma different quantities of bradykinin were added. Table 3 shows that, when 1.5 µg of bradykinin was used, a maximum of about 50 % of the substance was recovered (? minutes incubation time) and that all the bradykinin added was inactivated after 10 minutes. Under the conditions chosen disulfiram did not inhibit the enzymatic inactivation of bradykinin added to rat plasma.

Experiments with fresh rat blood from both normal rats and rats treated with disulfiram were also done (disulfiram orally by stomach tube,

about 50 mg/100 g in suspension twice at one day intervals and the experiment on the fourth day) Under these conditions padutin was again unable to release detectable quantities of kinins. Contrary to what was observed with "stable" rat plasma with kininase, small, but significant, amounts of kinin were found after incubation with trypsin, and in blood from normal rats as well as that from disulfiram treated rats.

Effect of disulfiram on the inactivation of bradykinin by erythrocyte kininase preparations from human blood and rat blood

The results in tables 4 and 5 show that disulfiram inhibited the inactivation of bradykinin by both human and rat erythrocyte kininase preparations An amount of disulfiram of 10 µg/ml was effective against the kininase activity of human erythrocytes, but the same concentration seemed rather to accelerate the effect of the rat kininase preparation. However if the concentration of disulfiram was increased to 100 µg/ml, the rat erythrocyte kininase preparation was also clearly inhibited.

Conclusion

The experiments show that padutin as well as human saliva released kinins in stable human plasma, but not in rat plasma. In stable rat plasma kinins were set free by adding trypsin. The results thus confirm the

Table 4

Inhibition by disulfiram of the inactivation of bradykinin by human erythrocyte kininase. Bradykinin 1.2 µg.
The figures refer to 1 15 ml incubation mixture with 1.0 ml tris buffer of pH 7.3.
F further details see text.

Disulfiram µg	Erythrocyte kininase preparation ml	% activity	after time in minutes
		100	104
	0.1	3	42
1000	0.1	100	98
		100	94
	0.1	3	32
100	0.1	50	75
		100	92
	0.1	6	32
10	0.1	18	32
		6	96
10		100	92
	0.1	20	28
10	0.1	70	31
		5	128

Enzyme preparation incubated with disulfiram for 34 minutes before addition of bradykinin.

Table 5

Inhibition by disulfiram of the inactivation of bradykinin by rat erythrocyte kininase.
Bradykinin 1.2 μg.
The figures refer to 1.15 ml incubation mixture with 0.1 ml tris buffer of pH 7.3
For further details see text.

Disulfiram μg	Erythrocyte kininase preparation ml	activity	after time in minutes
100		100	90
	0.1	Inactivation	in a few minutes
100	0.1	40	74
10		100	40
	0.1	3	21
10	0.1	More rapid inactivation than without disulfiram	

observations of FASCIOLO & HALVORSEN (1964) on rat plasma. The results obtained suggest that the method of AMUNDSEN, NUSTAD & WAALER (1963) yields a stable plasma substrate not only from human blood, but also from rat blood.

Under the conditions used disulfiram did not inhibit the inactivation by plasma kininase, either in human plasma or in rat plasma. When the inhibition by disulfiram of CPB in salt solution was tested on synthetic bradykinin as substrate, an effect was observed at pH 5.8, but not at the physiological pH of 7.3 The fact that larger concentrations of the enzyme had to be used at the lower pH value was consistant with the results of ERDÖS & LEWIS (1962). They showed that kininases from dog and ox plasma were inhibited at slightly acid pH values, between 6 and 7

Our experiments clearly demonstrate the high kininase activity in rat plasma compared with human plasma. The kinins released by incubation with trypsin were inactivated so quickly that only traces could be detected. To estimate any inhibition by disulfiram of the rat plasma kininase, considerable quantities of bradykinin accordingly had to be added to the plasma samples.

Experiments with fresh blood from normal rats showed that under such conditions disulfiram did not inhibit the plasma kininase either. Padutin did not release any detectable kinin in fresh blood from normal rats or rats treated for some days with disulfiram.

Disulfiram proved more effective against erythrocyte kininase than against plasma kininase. When enzyme preparations from human blood or from rat blood were tested in buffer solution at pH 7.3 and with bradykinin as substrate, disulfiram inhibited the inactivation of the kinin at

a concentration of 100 μg/ml. Previously ERDÖS, RENFREW, SLOANE & WOHLER (1963) found that the kininase in human plasma differed from the erythrocyte enzyme in its behaviour toward various inhibitors. Further AMUNDSEN (unpublished results) made the observation that urgocyton, a material isolated from liver (AMUNDSEN, WAALER, DEDICHEN, LALAND, LALAND & THORSDALEN) inhibited the kininase from human erythrocytes much more strongly than that from human plasma.

Summary

Experiments with stable human plasma substrate, "stable" human plasma substrate with kininase, stable rat plasma substrate, "stable" rat plasma substrate with kininase and fresh rat blood specimens were carried out to investigate whether disulfiram (= tetraethylthiuram disulphide) inhibited the inactivation of kinins by plasma kininase. Bradykinin was used as substrate in experiments with erythrocyte kininase. Padutin ® (hog pancreas kallikrein) or human saliva was used for releasing kinins from human plasma, trypsin those from rat plasma and rat blood. Under the conditions chosen no inhibition of the plasma kininases could be detected. When the effect of disulfiram on another Zn metalloenzyme, carboxypeptidase B (CPB) was tested in salt solution, with bradykinin as substrate, a strong inhibition was observed at pH 5.8 but not at 7.3.

In the inhibition experiments with "stable" rat plasma with kininase, the extremely high kininase activity of rat plasma, compared with that of human plasma, made it necessary to add bradykinin to the plasma samples. The kinins released by trypsin were inactivated so rapidly that only traces of kinins could otherwise be detected.

When the effect of disulfiram on the inactivation of synthetic bradykinin by erythrocyte kininase was tested, an inhibition of the enzyme was observed, both for human erythrocyte kininase and for rat erythrocyte kininase. The incubations were carried out in tris buffer at pH 7.3, and concentrations of disulfiram of 10 μg/ml or more proved effective against human erythrocyte kininase, 100 μg/ml or more against rat erythrocyte kininase.

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Depressor Effect of Padutin ® (Hog Pancreas Kallikrein) in Rats treated with Disulfiram

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Hog pancreas kallikrein (padutin ®) given intravenously to rats caused no fall in blood pressure, nor could a decrease in kallidinogen content of blood be demonstrated (HALVORSEN, personal communication, 1964). FASCIOLO & HALVORSEN (1964) explained the lack of effect in rats as due to "species specificity" If the use of that term is restricted to a failing drug receptor interaction, it should be remembered that a powerful enzymatic inhibition or inactivation of the agonist, or both, must also be considered possible causes of the absence of effect. The kallikrein itself might be affected, the kinin released, or both. As pointed out by HALVORSEN (personal communication) and also by BRISEID JENSEN, RINVIK & VENNERÖD (1965) the kininase activity of rat plasma is high compared with that of human plasma, a fact suggesting that the enzyme could be at least partly responsible for the inactivity of padutin. However the observation of HALVORSEN (unpublished results), that the kallidinogen content of rat blood did not fall in response to padutin injections, pointed to a true species specificity or to an enzymatic inhibition of padutin itself The use of a kininase inhibitor should provide more information on the subject. An inhibitor of sufficient strength would render padutin active if the high kininase level in rats were the main cause of its lack of activity On the other hand, absence of effect of the inhibitor would indicate species specificity or enzymatic inhibition of padutin, or both, as probable causes.

In our work, dimercaprol (BAL) 1 10-phenanthroline, and urgocyton were tested as kininase inhibitors in rat blood pressure experiments. Some experiments on capillary permeability inhibition by urgocyton in guinea pigs were also carried out. Dimercaprol was found by ERDÖS & WOHLER

(1963) to be a strong *in vivo* inhibitor of kininase in the guinea pig 1 10-phenanthroline was active *in vitro* in sera from different animals (ERDÖS, RENFREW SLOANE & WOHLER 1963) and urgocyton, a material isolated from liver by AMUNDSEN, WAALER, DEDICHEN, LALAND LALAND & THORSDALEN, was found active *in vitro* against the kininase of human erythrocytes (AMUNDSEN, personal communication, 1964) Disulfiram (tetraethylthiuram disulphide) was also tested for its possible action on the padutin effect on rat blood pressure. Disulfiram is known to be an inhibitor of the zinc enzyme, alcohol dehydrogenase of yeast (VALLEE 1960) Previous *in vitro* experiments had shown that disulfiram inhibited neither the Zn enzyme carboxypeptidase B nor the kininases present in human plasma or rat plasma at physiological pH but did inhibit those in human and in rat erythrocytes (BRISEID JENSEN, RINVIK & VENNERÖD 1965).

Technique

Rat blood pressure experiments

Male rats weighing 250–390 g were anaesthetized with a solution of barbitone sodium 4% w/v and 1% w/v pentobarbitone sodium of this, 0.4 ml/100 g was injected intraperitoneally The trachea was cannulated, and artificial respiration was applied in most experiments. Blood pressure was recorded from a carotid artery (heparin in the polythene cannula) with a mercury manometer and injections of the test substances were made into the two saphenous veins.

The effects of bradykinin, padutin, and trypsin were observed before and after injection of dimercaprol, 1 10-phenanthroline or urgocytone. Acetylcholine served as control substance.

Disulfiram was given orally by stomach tube in a suspension of 7.5 g disulfiram, 1 7 g carboxymethylcellulose and water to 100 ml. Two ml (150 mg) were given each time and in most experiments twice at an interval of one day (about 100 mg/100 g in all). On the fourth day the animals were used for the tests. On some occasions 3 or 4 doses of disulfiram were given, nd then over a correspondingly longer period.

Experiments were also carried out in which disulfiram was given on 3 subsequent days and the rats tested about 3 hours after the last dose.

Capillary permeability tests

Unanaesthetized guinea pigs. The method of MILES & MILES (1952) was used, largely with the minor modifications previously described (BRISEID JENSEN, VENNERÖD & DYRUD 1963).

Anaesthetized rats The rats were anaesthetized, as in the rat blood pressure experiments described above, and 0 15 ml of a 1% solution of Evans blue in water was injected intravenously into a femoral vein. Otherwise the technique was as in the guinea pig experiments.

Materials.

Acetylcholine chloride, crist., Hoffmann – La Roche & Co., A.G Basel, Switzerland.

Dimercaprol (= BAL) = 2,3-Dimercapto-1-propanol, für Komplexometrie, Fluka, A.G Buchs SG., Switzerland.

Bradykinin (synthetic) in ampoules of 100 µg/ml, Sandoz, A.G., Basel, Switzerland.

Histamine di-HCl, Light & Co Ltd., Colnbrook, England.

PADUTIN ® (hog pancreas kallikrein) Ampoules of 10 Biol. Units, Bayer A.G Leverkusen, Germany

1 10-*Phenanthroline*. o-Phenanthrolinium chlorid, Merck, A.G Darmstadt, Germany

Disulfiram. (= tetraethylthiuram disulphide) Preparation 1 synthetised in the Department of Pharmaceutical Chemistry the Pharmaceutical Institute, University of Oslo. Preparation 2, Farmaceutisk Industri, A.S., Oslo Norway

Trypsin (2 × crystalline), Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Urgocyton. Material isolated from ox liver Batch LO7A and batch U6. Yields respectively 2 g and 1.8 g/kg liver Preparations not dialysed. Batch SL 7 NS and batch SL 6 NS. Yields respectively 1.3 and 0.6 g/kg liver Preparations dialysed. Nyegaard & Co., A.S., Oslo, Norway

Results

A. *Rat blood pressure*

I *Normal rats* Padutin was injected intravenously into 15 rats at doses ranging from 4 to 12 units. The initial blood pressure levels ranged from 110 mm Hg to 140 mm Hg, and the rats reacted with significant blood pressure falls to acetylcholine doses of 10–20 ng and bradykinin doses of 0.3–0 6 µg. In a few rats padutin had some hypotensive effect, but either the blood pressure falls occurred with the first dose only or the falls were smaller at a higher dose level and were accordingly judged unspecific. (In one single rat with an extremely high blood pressure level, 150 mm Hg, padutin produced a depressor effect rather similar to that seen in disulfiram-treated rats. The blood pressure falls were not potentiated by dimercaprol) The experiments thus confirmed the unpublished observations of HALVORSEN, who injected padutin in doses up to 20 units without effect. To discover whether the presence of kininase in the padutin preparation might cause the lack of depressor effect, an experiment was carried out in which parallel tests of untreated padutin and padutin heated at 62° for 30 minutes were compared. No difference in effect could be demonstrated

II *Rats treated with dimercaprol* Intravenous doses of dimercaprol of 2 to 5 mg strongly potentiated the hypotensive effect of bradykinin. When the enzyme inhibitor was injected it caused an immediate fall in blood pressure. When the blood pressure had adjusted itself to a level near the

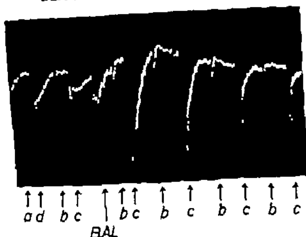


Fig. 1 Rat blood pressure.
Potentiation by dimercaprol (= BAL) of the bradykinin effect.
No effect on acetylcholine.
Blood pressure level 90 mm Hg.
Rat weight 300 g.

Acetylcholine	(a)	20 ng
–	(b)	40 –
Bradykinin	(c)	0.3 µg
	(d)	0.6
BAL		5 mg

initial one, bradykinin was injected. In the experiment illustrated in fig. 1 the first bradykinin dose was given about 4 minutes after the dimercaprol injection. The potentiation of the hypotensive effect disappeared fairly quickly and was not demonstrable after about 25 minutes, a duration of effect also noticed in other experiments. The effect of acetylcholine was the same before and after injection of dimercaprol. If another dose of dimercaprol was injected, the increase in bradykinin effect recurred and was usually more pronounced, as judged by the duration of effect.

Dimercaprol did not render padutin active. In the experiment illustrated by fig. 2 a dose of 5 mg dimercaprol strongly potentiated trypsin, but padutin was left unaffected. The transient increase in blood pressure level seen in fig. 2 after dimercaprol was observed in several experiments. The potentiation of trypsin was of the same duration as that of bradykinin, about 25 minutes.

III *Rats treated with 1,10-phenanthroline or urgocyton.*

1,10-phenanthroline was injected in single doses ranging from 100 to 300 µg. Higher doses seemed too toxic, and in one experiment 200 + 200 µg phenanthroline injected at an interval of about 90 minutes killed the rat. No clear potentiation of the bradykinin effect could be demonstrated even though padutin sometimes showed a depressor effect. The effect was, however irregular and difficult to reproduce.

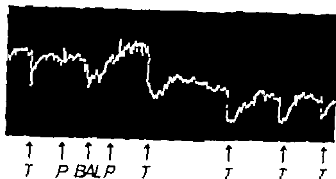


Fig. 2. Rat blood pressure
Potentiation by dimercaprol (= BAL) of the effect of trypsin.
N effect on padutin.
Initial blood pressure level 90 mm Hg.
Rat weight 300 g.
Trypsin (T) 35 µg
Padutin (P) 12 U
BAL 5 mg

Four batches of *urgocytan* were examined, but *none of them increased* the depressor effect of bradykinin in doses up to about 10 mg/100 g.

IV *Rats treated with disulfiram* When rats were pretreated with disulfiram, as described above (see *Technique*) *padutin regularly had a* hypotensive effect. The kallikrein preparation was given to 17 animals, all of which reacted with more or less pronounced falls in blood pressure. The effect was rather feeble, large padutin doses being required (3–12 units). *The best effect was obtained when 3 doses of disulfiram of 150 mg* were spaced over a period of 3 days and the animals were used for experiments about 3 hours after the last dose In fig. 3 part of such an experiment is illustrated Injections of 3 6, and 12 units of padutin caused graded blood pressure falls. The hypotensive effects of padutin and trypsin were compared roughly quantitatively Twelve units of padutin resulted in a blood pressure fall of the same order of size as that caused by 10–20 µg trypsin. The falls, however were often more long-lasting than those due to trypsin and more like a temporary depression in blood pressure. Doses of *mepyramine, which almost completely abolished the hypotensive* effect of 0.5 µg histamine, did not affect the action of padutin.

In disulfiram treated rats dimercaprol also potentiated the effect of bradykinin but not that of padutin. Fig. 4 illustrates an experiment in which the effects of 4 and 8 units of padutin were small both before and after 2 mg of dimercaprol, but bradykinin was strongly potentiated The figure also shows that the potentiation of bradykinin disappeared at the same rate as in untreated rats after about 25 minutes the effect was normal



Fig. 3. Depressor effect of padutin on rat treated with disulfiram (= tetraethylthiuram disulphide).
Blood pressure level: 80 mm Hg.
Rat weight, 260 g.
Disulfiram, 3 doses of 150 mg orally spaced over 3 days.
Padutin (a): 12 U.
(b) 6 U
(c) 3 U
Bradykinin (d) 0.3 μg



Fig. 4 Blood pressure of rat pretreated with disulfiram (= tetraethylthiuram disulphide).
Potentiation by dimercaprol (= BAL) of the bradykinin effect.
No effect on Padutin.
Blood pressure level 85 mm Hg.
Rat weight 320 g.
Disulfiram 2 doses of 150 mg orally
Bradykinin (a) 0.3 μg
Padutin (b) 4 U
(c) 8 U
BAL 1.2 mg.



Fig. 2. Rat blood pressure
Potentiation by dimercaprol (= BAL) of the effect of trypsin.
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Fig. 3 Depressor effect of padutin on rat treated with disulfiram (= tetraethylthiuram disulphide).

Blood pressure level 80 mm Hg.
Rat weight 260 g.
Disulfiram 3 doses of 150 mg orally spaced over 3 days.
Padutin (a) 12 U
– (b) 6 U
– (c) 3 U
Bradykinin (d): 0.3 μg

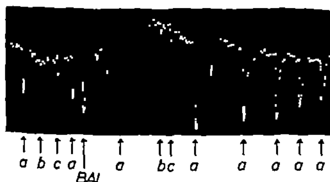


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Potentiation by dimercaprol (= BAL) of the bradykinin effect.
No effect on Padutin.

Blood pressure level 85 mm Hg.
Rat weight 320 g.
Disulfiram 2 doses of 150 mg orally
Bradykinin ( ) 0.3 μg
Padutin (b) 4 U
(c) 8 U
BAL 2 mg.



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Potentiation by dimercaprol (= BAL) of the effect of trypsin.
No effect on padutin.
Initial blood pressure level 90 mm Hg
Rat weight 300 g.
Trypsin (T) 33 µg
Padutin (P) 12 U
BAL 5 mg

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Fig. 3. Depressor effect of padutin on rat treated with disulfiram (= tetraethylthiuram disulphide).
Blood pressure level: 80 mm Hg.
Rat weight 260 g.
Disulfiram 3 doses of 150 mg orally spaced over 3 days.
Padutin (a): 12 U
– (b): 6 U
– (c) 3 U
Bradykinin (d) 0.3 µg

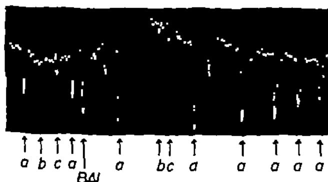


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No effect on Padutin.
Blood pressure level 85 mm Hg.
Rat weight 320 g.
Disulfiram 2 doses of 150 mg orally
Bradykinin (a) 0.3 µg
Padutin (b): 4 U
– (c) 8 U
BAL 2 mg.

B *Capillary permeability*

I. *Padutin* *Intradermal* injections of padutin increased capillary permeability in both rats and guinea pigs. Doses of 100 mU/100 g in rats were found roughly to give skin spots of the same colour intensity and same order of size as those caused by 150 ng of bradykinin. In guinea pigs padutin was relatively more effective, doses of 6 mU/100 g corresponding to 75 ng of bradykinin.

II *Urgocyton* *Intradermal* injections of 50 to 200 μg/100 g of batch SL6NS and SL7NS of urgocyton into guinea pigs increased the capillary permeability effect of 30 to 100 ng/100 g of bradykinin injected in the same solution.

Intravenous injections of about 800 μg urgocyton/100 g guinea pig (batch SL6NS) *seemed to affect neither the action of bradykinin in* increasing capillary permeability nor that of histamine Trypsin, which was used to control the technique, inhibited both bradykinin and histamine markedly at a dose of 0.3 mg/100 g. The experiments were designed as described below Soon after the injection of Evans blue, two doses of bradykinin and two doses of histamine were injected intradermally in random order The bradykinin colour spots appeared in the course of 2–3 minutes and the histamine spots in the course of 3–4 minutes. Twenty minutes after the injection of Evans blue, urgocyton (or trypsin) was applied and then the substances increasing capillary permeability were again injected, as described above. The whole duration of the experiment was about 1 hour Bradykinin and histamine were found active at a dose range of respectively 5 to 50 ng and 8 to 50 μg/100 g guinea pig.

Discussion

Our experiments were carried out to investigate whether or not kininase inhibitors would cause an effect of hog pancreas kallikrein (padutin ®) on rat blood pressure. The use of dimercaprol showed that the strong kininase activity known to be possessed by rat plasma (HALVORSEN, personal communication BRISEID JENSEN RINVIK & VENNERØD 1965) is probably not responsible for the inactivity of padutin Doses of dimercaprol that strongly potentiated the depressor effect of bradykinin and also of trypsin did not render padutin active. The results were consistent with the *in vitro* observations of FASCIOLO & HALVORSEN (1964) and also of BRISEID JENSEN, RINVIK & VENNERØD (1965) who found padutin unable to release kinin from stable rat plasma.

The extremely strong kininase activity of rat plasma was probable the reason for the inability of the kininase inhibitor 1 10-phenanthroline to potentiate bradykinin. The short duration of the dimercaprol effect could

possibly also reflect the strong kininase activity of the rat. All potentiation had vanished after about half an hour whereas ERDÖS & WOHLER (1963) observed an inhibition of several hours duration for the same dimercaprol doses when guinea pigs were used. As to the failing ability of urgocyton in inhibiting the plasma kininase in rat blood pressure experiments, it should be pointed out that AMUNDSEN (personal communication) found urgocyton far less active against human plasma kininase than against erythrocyte kininase.

Disulfiram, which had previously proved unable to protect bradykinin *in vitro* at a physiological pH against carboxypeptidase B or plasma kininase (BRISEID JENSEN, RINVIK & VENNERÖD 1965) rendered padutin hypotensive in rats. The effect was somewhat weak, doses of 3 to 12 units being required. As the substance had to be given before the experiments, no estimate of a possible influence on the bradykinin effect, and so on the kininase level, was possible. However the doses of bradykinin necessary in the disulfiram-treated rats were of the normal order On considering at the same time the above-mentioned inability of padutin to release kinins *in vitro* in kininase-free medium and to cause blood pressure fall in presence of the strong kininase inhibitor dimercaprol, it seems unlikely that the main action of disulfiram was through plasma kininase inhibition. A more likely assumption is that padutin in rats has some depressor effect of its own and that disulfiram inhibits the plasma enzyme inactivating padutin. The ability of padutin to induce increased capillary permeability by local application in the skin gives support to such a view

Even if no kinin-releasing effect of padutin could be detected *in vitro* (BRISEID JENSEN, RINVIK & VENNERÖD 1965) the possibility cannot be excluded that it had a slight effect *in vivo* It should be kept in mind that disulfiram inhibited the kininase activity of rat erythrocytes and that such an effect might contribute to the depressor effect of padutin.

Summary

Padutin ® (hog pancreas kallikrein) injected intravenously into rats caused no fall in blood pressure. Kininase inhibitors were used to investigate whether or not the high kininase activity of rat plasma, compared with that of human plasma, might contribute to the lack of effect. Dimercaprol, urgocyton and 1 10-phenanthroline were examined for their abilities to increase the depressor effect of bradykinin, but only dimercaprol proved effective within the dose ranges used. Dimercaprol also potentiated the depressor effect of trypsin, but did not render padutin active.

When disulfiram (tetraethylthiuram disulphide) was given to the rats before the experiments, padutin became hypotensive, but large doses of

the kallikrein preparation were required (1–4 U/100 g) and the blood pressure falls were sometimes small. The experiments suggested that disulfiram did not act through an inhibition of plasma kininase. It seems more likely that padutin has an inherent depressor effect not connected with kinin release and that disulfiram might possibly release such an effect through inhibition of the enzyme inactivating padutin.

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Fate of Mepivacaine in the Body
I. Whole-Body Autoradiographic Studies of the Distribution of ^{14}C labelled Mepivacaine in Mice

By

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Mepivacaine[1]), (N methyl-2-piperidine carboxylic acid)-2, 6'-dimethyl anilide-hydrochloride, has been shown to be a valuable local anaesthetic (Ekenstam, Egnér, Ulfendahl, Dhunér & Oljelund 1956 Dhunér, Oljelund & Aagesen 1957 Dam & Guldman 1957 Bezzenberger & Stellmach 1957 Berling 1958 Sheffield, Kennedy & Dornette 1964) There has been no report in the literature on the physiological fate or the biotransformation of the compound. It has, however been shown that it is readily metabolized or excreted, since Luduena, Hoppe, Coulston & Drobeck (1960) could administer one half of the LD50 every half hour with little cumulative effect.

The aim of our investigation was to study the physiological disposition of mepivacaine after intravenous and subcutaneous injection. The mepivacaine had been labelled with ^{14}C, and the distribution in the body was studied by autoradiography

Methods

^{14}C-labelled mepivacaine was synthetized by AB Nobelkrut, Bofors, Sweden as indicated below Pyridine 2-carboxylic acid-2, '6'-dimethylanilide was prepared from pyridine-2 carboxylic acid and 2,6 dimethylaniline. The pyridine ring was then hydrogenated. The product, piperidine-2-carboxylic acid-2, '6'-dimethylanilide, was then N-methylated in ethanol solution by reaction with ^{14}C-methyliodide. The specific activity of the mepivacaine was 2.34 mC/g. The mepivacaine was dissolved in physiological saline to a 0.4% (w/v) solution.

1) Carbocaine ® and scandicaine ® are the registered trade-marks of mepivacaine.

Male and female white mice were used. Four series of experiments were performed. In the first series male mice weighing approximately 25 g were injected in the tail vein with 30 mg/kg (corresponding to 70 μC/kg) of ^{14}C-mepivacaine. The injection volume was 0.3 ml and the injection time approximately 2 minutes. Animals were killed at 2, 5 (2 animals), 20 minutes, 1 hour (2 animals) 4 hours (2 animals), 24 hours, 48 hours and 96 hours after the injection.

In a second series male adult mice weighing approximately 25 g were injected subcutaneously with 30 *mg/kg (corresponding to 70 μC/kg) of* ^{14}C-mepivacaine. Animals were killed at 5 minutes, 20 minutes, 1 hour 4 hours, 24 hours, 48 hours, 72 hours and 96 hours after the injection.

Mice of a third series were used to observe the placental passage of the drug. Pregnant mice (approximately 40 g body weight and 2 days before delivery) were injected in the tail vein with 40 mg/kg (corresponding to 94 μC/kg) of ^{14}C-mepivacaine. Animals were killed at 5 minutes, 20 minutes, 1 hour 4 hours and 24 hours (2 animals) after the injection.

All the animals were lightly anaesthetized with ether and then killed by freezing in an acetone-dry ice mixture. The sectioning and autoradiographic exposure were then performed as described by ULLBERG (1954 & 1958).

Mice of a fourth series were used to examine selected tissues for metabolic products of mepivacaine. ^{14}C-mepivacaine (30 mg/kg corresponding to 70 μC/kg) was administered *via* a tail vein to male mice weighing approximately 25 g. The brain, submaxillary glands, liver and kidney were removed from mice 5 20 and 60 minutes after the injection. The intestines were removed 60 minutes after the injection. The organs were homogenized in glass-homogenizers with phosphate buffer at pH 7 4 The homogenate was transferred to extraction tubes and brought to pH 9 by adding NH_4OH. The contents of the tubes were then extracted continuously with ether The ether phase was reduced in volume under nitrogen to 5 ml. Radioactivity was determined in the ether and water phases by liquid scintillation counting. The scintillation solution was composed of 7 ml toluene containing 5 g 2.5-diphenyloxazole/1 toluene and 3 ml absolute ethanol. The nature of the radioactive compounds in the ether and the water phases was investigated by thin-layer chromatography The solvent systems for thin-layer chromatography were ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5) (v/v) and chloroform-methanol (2 1). Silica gel plates were prepared as described by STAHL (1962). After drying the plate by allowing it to stand in the air radioactive compounds were located by exposing the plate to Kodak No Screen X-ray film for two weeks.

Results

Tissue distribution

Intravenous injection.

The distribution pictures of radioactivity at 5, 20 minutes and 1 hour after intravenous injection are shown in figs. 1 2 and 3 Mepivacaine rapidly left the blood and was *concentrated in the tissues. The tissues with* the highest concentration 5 and 20 minutes after the injection were brain salivary glands, liver kidney bone marrow and gastric and intestinal mucosa.

The highest concentration was seen in excretory organs such as tl

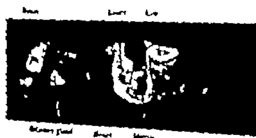


Fig. 1. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 5 minutes after intravenous injection of ^{14}C-mepivacaine. Sagittal section through the centre body of the animal. Note high concentrations in the heart, salivary gland and stomach mucosa.



Fig. 2. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 20 minutes after intravenous injection of ^{14}C-mepivacaine. High concentrations are observed in the stomach contents, kidney and urinary bladder.



Fig. 3. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 1 hour after intravenous injection of ^{14}C-mepivacaine. Most of the radioactivity is seen in the intestinal contents and the urinary bladder. The radioactivity in the brain is low.

kidney, urinary bladder, liver and gall bladder in those animals killed 1 hour and more after injection.

The brain, especially the grey matter, rapidly took up radioactivity. The highest concentration was observed in the animals killed 2 and 5 minutes after the injection. The radioactivity left the brain more quickly than other tissues. The level in the brain was low 1 hour after injection and approached the blood level.

The submaxillary glands, the kidney and the liver were the organs

Male and female white mice were used. Four series of experiments were performed. In the first series male mice weighing approximately 25 g were injected in the tail vein with 30 mg/kg (corresponding to 70 µC/kg) of ^{14}C-mepivacaine. The injection volume was 0.3 ml and the injection time approximately 2 minutes. Animals were killed at 2, 5 (2 animals) 20 minutes, 1 hour (2 animals), 4 hours (2 animals), 24 hours, 48 hours and 96 hours after the injection.

In a second series male adult mice weighing approximately 25 g were injected subcutaneously with 30 mg/kg (corresponding to 70 µC/kg) of ^{14}C-mepivacaine. Animals were killed at 5 minutes, 20 minutes, 1 hour 4 hours, 24 hours, 48 hours, 72 hours and 96 hours after the injection.

Mice of a third series were used to observe the placental passage of the drug. Pregnant mice (approximately 40 g body weight and 2 days before delivery) were injected in the tail vein with 40 mg/kg (corresponding to 94 µC/kg) of ^{14}C-mepivacaine. Animals were killed at 5 minutes, 20 minutes, 1 hour 4 hours and 24 hours (2 animals) after the injection.

All the animals were lightly anaesthetized with ether and then killed by freezing in an acetone-dry ice mixture. The sectioning and autoradiographic exposure were then performed as described by ULLBERG (1954 & 1958).

Mice of a fourth series were used to examine selected tissues for metabolic products of mepivacaine. ^{14}C-mepivacaine (30 mg/kg corresponding to 70 µC/kg) was administered *via* a tail vein to male mice weighing approximately 25 g. The brain, submaxillary glands, liver and kidney were removed from mice 5 20 and 60 minutes after the injection The intestines were removed 60 minutes after the injection. The organs were homogenized in glass-homogenizers with phosphate buffer at pH 7 4 The homogenate was transferred to extraction tubes and brought to pH 9 by adding NH_4OH. The contents of the tubes were then extracted continuously with ether The ether phase was reduced in volume under nitrogen to 5 ml. Radioactivity was determined in the ether and water phases by liquid scintillation counting. The scintillation solution was composed of 7 ml toluene containing 5 g 2,5-diphenyloxazole/l toluene and 3 ml absolute ethanol. The nature of the radioactive compounds in the ether and the water phases was investigated by thin-layer chromatography The solvent systems for thin-layer chromatography were ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5) (v/v) and chloroform-methanol (2 1). Silica gel plates were prepared as described by STAHL (1962). After drying the plate by allowing it to stand in the air radioactive compounds were located by exposing the plate to Kodak No Screen X-ray film for two weeks.

Results

Tissue distribution

Intravenous injection.

The distribution pictures of radioactivity at 5 20 minutes and 1 hour after intravenous injection are shown in figs. 1 2 and 3 Mepivacaine rapidly left the blood and was concentrated in the tissues. The tissues with the highest concentration 5 and 20 minutes after the injection were brain, salivary glands, liver kidney bone marrow and gastric and intestinal mucosa.

The highest concentration was seen in excretory organs such as the

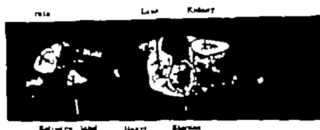


Fig. 1 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 5 minutes after intravenous injection of ^{14}C-mepivacaine. Sagittal section through the entire body of the animal. Note h gh concentrations in the brain, salivary gland and stomach mucosa.



Fig. 2 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 20 minutes after intravenous injection of ^{4}C-mepivacaine. High concentrations are observed in the stomach contents, kidney and urinary bladder

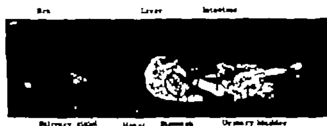


Fig 3 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 1 hour after intravenous njectio of ^{4}C mepivacaine. Most of the radioactivity is seen in the intestinal contents and the urinary bladder The radioactivity in the brain is low

kidney urinary bladder liver and gall bladder in those animals killed 1 hour and more after injection.

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The submaxillary glands, the kidney and the liver were the organs

14a



Fig. 4 Autoradiogram showing the distribution of radioactivity (light areas) in a pregnant mouse 1 hour after injection of ^{14}C-mepivacaine. Not the high concentration of radioactivity in the gall bladder The radioactivity in the foetuses is low

showing the highest concentration of radioactivity The level in the liver decreased rather rapidly probably owing to biliary excretion (fig. 4). The activity in the submaxillary glands was low in the animals killed 4 hours after injection. A high concentration of radioactivity was also seen in the adrenal medulla.

The kidney and urinary bladder showed a high concentration soon after injection. The activity was also high in the gastrointestinal tract. This was probably due to secretion via the gastric mucosa (fig. 1–3) and the bile (fig. 4)

No retention of ^{14}C (mepivacaine or its metabolites) could be observed in any tissue. Four hours after injection most of the radioactivity was present in the intestinal contents. In animals killed 24 hours and later after injection radioactivity was seen only in the intestinal contents and in the urinary bladder

Experiments were also performed on pregnant mice to observe placental penetration and uptake of the drug by the foetus. The concentration of mepivacaine or its metabolites on both was always lower in the foetal than in the maternal tissues (fig. 4) The radioactivity was not selectively localized in any of the tissues of the foetus and left the foetus as the concentration in the mother decreased

Subcutaneous injection.

In the animals killed 5 minutes after injection little activity was noted in the tissues. Mepivacaine was absorbed slowly and remained mainly at the injection site. The tissues showing activity were kidney submaxillary glands and brain The autoradiograms taken 20 minutes after subcutaneous injection showed a distribution picture similar to that obtained 5 minutes after an intravenous injection (fig. 5). The highest activity however was still at the site of injection. Appreciable activity was

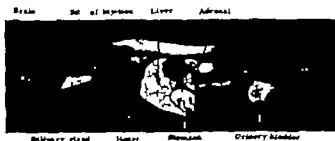


Fig. 5. Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 20 minutes after subcutaneous injection of ^{14}C-mepivacaine. The highest concentration is seen at the site of injection and in the liver salivary gland, stomach, kidney and adrenal medulla. The radioactivity in the brain is rather low

observed in brain, liver kidney salivary gland, urinary bladder and stomach contents. The brain did not reach the same high level of radioactivity as after intravenous injection, although the amount of radioactivity was substantial.

The pictures after 20 minutes and 1 hour were much alike. After 1 hour the radioactivity in the liver kidneys and intestinal contents dominated the picture.

After 4 hours, the highest concentration was observed in the intestinal contents and the kidney Little or no activity was then observed at the site of injection.

By 24 hours mepivacaine or its breakdown products or both were only seen in the intestinal contents. In the animals killed at 2, 3 and 4 days after administration only traces of radioactivity or none could be observed.

Examination of tissues for radioactive compounds

Table 1 shows the radioactivity present in the ether and the water phases of extracts from various organs. The extraction of unchanged mepivacaine into the ether phase was almost 100 %. The radioactivity in the brain was ether-soluble at all times after administration, and chromatographic investigation showed only one radioactive compound in the brain. This compound had the same R_f value as mepivacaine.

Extracts from liver and kidney indicate that mepivacaine was rapidly metabolized to several less lipid-soluble metabolites. In the animals killed 60 minutes after injection, the kidney content of metabolites was more than 10 times as high as the amount of unchanged drug. Thin-layer chromatography showed that the radioactivity in the ether phase was associated with material having R_f value as unchanged mepivacaine.

The radioactivity in the water phase was due to at least 4 different com-

Table 1

Radioactivity in the ether and the water phases from the brain, the salivary glands, the liver, the kidney and the intestines of mice at various times after intravenous injection of ^{14}C Mepivacaine. Percentage of injected radioactivity in the tissues.

Tissues	Time after injection					
	5 minutes		20 minutes		60 minutes	
	Ether	Water	Ether	Water	Ether	Water
Brain	2.4	0	2.4	0	0.5	0
Submaxillary glands	0 6	0	0 7	0.3	0 5	0.1
Liver	7.5	4.2	4.5	4.8	2.8	7.5
Kidney	3.2	0.3	1 7	1 7	0.5	5.2
Intestines	–	–	–	–	3.5	9.8

pounds. Attempts at identification of these breakdown products are in progress.

Discussion

The examination of tissues for radioactive compounds showed that mepivacaine was rapidly metabolized to some less lipid-soluble compounds. The autoradiograms taken later therefore showed the presence of unchanged mepivacaine as well as its metabolites It must be pointed out that subsequent investigations have shown a considerable part of the injected mepivacaine to be demethylated with loss of the ^{14}C labelled methyl group

The highest accumulation of radioactivity was in the liver The radioactivity observed there 20 minutes after injection and later was to a large extent due to degradation products of mepivacaine. The uptake in the liver is thus probably a reflection of the metabolism of the compound by this organ. High radioactivity was observed in the bile, indicating considerable biliary excretion.

High uptake of mepivacaine was observed in the brain shortly after intravenous injection This rapid uptake was followed by a rapid decline. Mepivacaine behaves in this respect like, e.g., thiobarbiturates (MARK, BURNS, CAMPOMANES, NGAI TROUSOF PAPPER & BRODIE 1952) and nicotine (APPELGREN HANSSON & SCHMITERLÖW 1963). Only unchanged mepivacaine was found in the brain. On the basis of our studies it is likely that a central nervous system reaction to accidental intravenous injection may occur during the first minute after the injection. On the other hand a reaction in the central nervous system due to overdoses or a specific sensitivity to mepivacaine after infiltration anaesthesia may not appear

until 5–20 minutes after the injection This is in agreement with the clinical findings of a sedative effect occurring in some patients within 5–10 minutes and disappearing after 30–60 minutes (SHEFFIELD KENNEDY & DORNETTE 1964)

Another organ accumulating mepivacaine to a high degree was the submaxillary glands. This accumulation is difficult to explain, but we have noticed it to occur with some basic drugs, whereas some acidic drugs are poorly accumulated there (HANSSON & SCHMITERLÖW 1961 a & b HANNGREN HANSSON, SVARTZ & ULLBERG 1963)

Excretion of mepivacaine or its metabolites or both into the stomach took place to a considerable extent. The drug shares this property with some other basic drugs, such as antipyrine, mecamylamine and aminopyrine (SCHANKER 1962)

The drug penetrated the placenta and showed a uniform distribution in the foetus. The foetal content was always lower than the material. This suggests simple diffusion of the drug through the placenta without active uptake by the foetus.

No specific retention of the drug in the tissues was observed. Some level of radiocarbon, however remained in the tissues 1 and 2 days after administration. Since demethylation has been observed to be one of the major metabolic routes for mepivacaine (HANSSON, HOFFMANN & KRISTERSON 1965), it is probable that this radioactivity does not represent mepivacaine or metabolites, but is the result of radiocarbon released by demethylation becoming involved in the general metabolism

Summary

The distribution of the local anaesthetic, mepivacaine, has been studied in mice. The compound was labelled with ^{14}C, which permitted autoradiographic study of its distribution Mepivacaine, its metabolites or both accumulated in the liver the kidney the salivary glands and the brain at short intervals after administration. Experiments on pregnant animals demonstrated little passage of radioactivity into the foetuses. Examination of tissues for radioactive compounds by thin-layer chromatography showed that mepivacaine was rapidly metabolized probably in the liver

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Fate of Mepivacaine in the Body
II. Excretion and Biotransformation

By

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(Received October 8, 1964)

In a previous report (KRISTERSON, HOFFMANN & HANSSON 1965) it was demonstrated that ^{14}C mepivacaine[1]), (N-methyl-2 piperidine-carboxylic acid)-2, 6'-dimethylanilide-hydrochloride, was rapidly distributed in the body after intravenous or subcutaneous administration. It was also found to be metabolized to several less lipid-soluble products. LUDUENA *et al* (1960) have suggested that mepivacaine is readily metabolized, since they could administer one half of the LD50 every half hour with little cumulative effect.

This report is of a quantitative study of the excretion and metabolism of mepivacaine in mice and rats, including some attempts to identify the breakdown products.

Methods

Synthesis of ^{14}C-Mepivacaine

^{14}C-methyl-labelled mepivacaine was synthetized by AB Nobelkrut, Bofors, Sweden, by the method already described (KRISTERSON, HOFFMANN & HANSSON 1965) The specific activity was 2 3 µC/mg.

Excretion into the faeces and urine

Adult male rats weighing 300 g were injected intravenously with 2 mg (4.6 µC) of ^{14}C-mepivacaine. They were then kept in metabolism cages for 72 hours, to collect urine and faeces.

Treatment of urine

Samples of 24-hour urine were taken some of them were adjusted to pH 1 by adding 0.5 N-HCl and the others adjusted to pH 9 by adding 0.5 N-NH_4OH. The

[1]) Carbocaine ® and scandicaine ® are the registered trademarks of mepivacaine.

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was determined on 0.1 ml of chloroform and water phases by liquid scintillation counting after adding 7 ml toluene containing 0.5 % DPO and 3 ml absolute ethanol.

β-glucuronidase hydrolysis.

Bile. One ml of a pooled sample from the first 6 hours was mixed with an equal volume of phosphate buffer pH 7. The sample was then incubated for 24 hours with 2500 units of bacterial β-glucuronidase (Sigma). Controls containing no enzyme were run in parallel.

Measured portions from each of these incubated bile samples were extracted three times with 10 ml of chloroform at pH 9. The water phases and the chloroform phases were reduced in volume under nitrogen and chromatographed.

Urine. Two ml were diluted with an equal amount of a phosphate buffer pH 7 containing 2500 units bacterial β-glucuronidase (Sigma). Incubation and extraction were performed as for the bile.

Respiratory radiocarbon dioxide.

Adult mice (body weight 25 g) were injected in the tail vein with 0.4 mg (1 μC) of the ^{14}C-mepivacaine solution. The animals were then placed in a plastic metabolic apparatus. The respiratory CO_2 was collected in a phenethylamine mixture and counted in a liquid scintillation counter as described by HANSSON & CLARK (1962).

Chromatography

Mepivacaine and its metabolites in bile and urine were separated by thin-layer chromatography.

Thin-layer silica gel plates of 250 μ thickness were prepared by the procedure of STAHL (1962). The solvent systems were chloroform-methanol (2 1) and ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5).

The radioactivity on the chromatograms was detected by autoradiography. The autoradiograms were prepared by allowing the chromatograms to remain in contact with X-ray film for suitable periods. Quantitative determination of the radioactive compounds separated on the thin-layer plates was performed. These areas were scraped with a razor blade into 20 ml liquid scintillation vials and counted as a suspension in 0.5 % DPO + 0.03 % POPOP + 4 % silica gel (Aerosil) in toluene (SNYDER & STEPHENS 1962).

Results

Excretion

The urinary excretion of radioactivity derived from intravenous injection of ^{14}C-mepivacaine is shown in table 1. There was a rapid appearance of radioactivity in the urine, approximately 15 % of the administered radioactivity within 1 hour in two rats. After 24 hours the total urinary excretion of radioactivity accounted for approximately 55 % of the dose. In succeeding intervals only a small percentage was excreted.

A small amount of the administered radioactivity appeared in the the maximum amount during the first 24 hours.

Table 1

Excretion of radioactivity in urine and faeces after intravenous injection of [14]C-mepivacaine.
Excretion expressed as percentage f dose administered. Mean value from three animals. Range in parentheses.

Time intervals (hours)	Urine	Faeces
0–24	54.1 (49 3–62.3)	2.9 (1.8–5 0)
24–48	3.5 (1.9– 5 0)	1.3 (0.6–1 7)
48–72	1.3 (0 6– 2.9)	0 1 (0 –0.2)
Total	59 1	4.3

The experiments with bile fistulae showed that in a 6 hour period 51 4–56 2 / of the administered radioactivity was excreted into the bile (table 2) It may be noted that between 27 5 and 32.4 / of the radioactivity was excreted within 30 minutes after the injection of [14]C mepivacaine.

Metabolism

Radioactive CO_2 in the expired air was collected from mice during an 8 hour period at intervals of 0–2, 2–4 and 4–8 hours. The results of these studies are shown in table 3 Between 10.5 and 11 4 / of the dose was excreted during the 8 hour period. The main part of this activity appeared during the first two hours after the intravenous injection of [14]C mepivacaine

Liver slices were incubated with radioactive mepivacaine in Warburg vessels. The compound was metabolized to a large extent in oxygen. Under anaerobic conditions mepivacaine was not metabolized, and some metabolism occurred in air In table 4 is shown the distribution of radioactivity in the chloroform, water and carbon-dioxide fractions of the

Table 2

Excretion f radioactivity in at bile for 6 hours after intravenous injection of [14]C-mepivacaine.

Rat No.	Per cent f dose excreted					
	0–30 min.	30–60min.	1–2 h.	2–4 h.	4–6 h.	Total
6	27 6	11 4	9 6	2.2	0 6	51 4
7	29 0	11 9	2.4	8 9	1 9	54 1
8	27.5	18.7	5 0	3.0	2.3	56 5
9	32.4	12.9	6.6	1 3	2.0	55 2

Table 3

Excretion of radioactivity into the respired air after intravenous injection of ^{14}C mepivacaine into mice

Mouse No.	Per cent ^{14}C in respiratory air			Total
	0–2 h	2–4 h	4–8 h.	
10	8.8	1.3	0.4	10.5
11	8.4	1.7	0.4	10.6
12	8.0	1.7	1.7	11.4

incubated liver slices. Measured portions of the water and chloroform phases were used for thin-layer chromatography

A 24-hour urine sample from rats receiving ^{14}C mepivacaine was extracted with ether and chloroform at pH 1 and pH 9 No radioactivity was extracted at pH 1 or only traces, but 17–32 % of urinary radioactivity could be extracted at pH 9 The same procedure was used for bile. Only traces could be extracted into ether or chloroform at pH 1 or pH 9 Mepivacaine itself was extracted to almost 100 % by either chloroform or ether at pH 9 Clearly the radioactivity excreted into bile and urine was to a large extent associated with metabolites.

Treatment of urine and bile with β-glucuronidase and their extraction at pH 9 with chloroform, showed that an appreciable amount of radioactivity was now chloroform-soluble. From the bile 80–82 % could be extracted into chloroform and from the urine 76–85 %.

Fig. 1 shows an autoradiogram of a thin-layer chromatogram of bile and of chloroform extracts of urine and liver slices. The spot with R_f = 0.81 is mepivacaine. The radioactive components found in the urine were also observed in the extract of liver slices The bile contained mainly water-soluble components, which stayed at the origin and contained only

Table 4

Distribution of radioactivity in various chemical fractions after incubation of liver slices Krebs-Henseleit Buffer in air 100 % O_2 and 100 % N_2. Mean values from two experiments.

Gas	Per cent radioactivity in various fractions			Total
	Chloroform phase	Water phase	$^{14}CO_2$	
Air	85.6	7.4	2.3	95.2
O_2	54.2	24.5	15.9	95.6
N_2	97.1	1.3	0.1	98.5

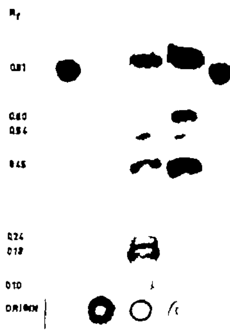


Fig. 1 Autoradiogram f thin-layer chromatogram of bile, the chloroform phase after extraction of urine from a rat injected with [14]C-mepivacaine and the chloroform phase after extraction of liver slices incubated with [14]C-mepivacaine. Solvent system ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5) (/v). Solvent front = 16 cm. Exposure time = 7 days. Columns from left to right Standard, mepivacaine Bile Urine Liver slices Standard, mepivacaine.

traces of unchanged mepivacaine The spot with R_f = 0 45 had the same R_f value as the p-hydroxyderivative of mepivacaine shown in fig. 4 Quantitative results are given in table 5

Table 5

Distribution of radioactivity between metabolites found in the bile nd the chloroform phases from urine nd incubated liver slices. The values are derived from the chromatogram shown in fig. 1

	Bile	Urine (chloroform phase)	Incubated liver slices (chloroform phase)
Mepivaca ne (R_f = 0.81)	–	56 0	84.5
R_f = 0 60	–		3.0
R_f = 0.54	0.1	1.6	0.8
R_f = 0.45	–	15.0	10.5
R_f = 0.24	–	–	0.2
R_f = 0.18	–	24.0	0.2
R_f = 0.10	–		0.4
Origin	99 9	3.4	0.2

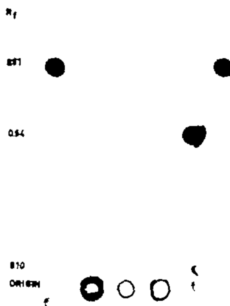


Fig. 2. Autoradiogram of thin-layer chromatogram of chloroform and water phases of bile before and after hydrolysis with β-glucuronidase. Solvent system ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5) (v/v). Solvent front = 16 cm. Exposure time = 7 days. Columns from left to right Standard mepivacaine Water phase before hydrolysis Chloroform phase before hydrolysis Water phase after hydrolysis Chloroform phase after hydrolysis Standard mepivacaine.

Fig. 2 shows an autoradiogram of a thin-layer chromatogram of the chloroform and water phases of extracted bile before and after treatment of the bile with β-glucuronidase. Before hydrolysis almost all the radioactivity was found in the water phase. After treatment with β-glucuroni-

Table 6

Distribution of radioactivity between metabolites found in the chloroform phase and the water phase from bile before and after hydrolysis with β-glucuronidase. The values are derived from the chromatogram shown in fig. 2.

	Percentage of total radioactivity	
	before hydrolysis	after hydrolysis
Chloroform phase		
R_f = 0.54	–	77.9
R_f = 0.10	–	2.8
Origin	1.3	3.3
Water phase		
Origin	98.7	

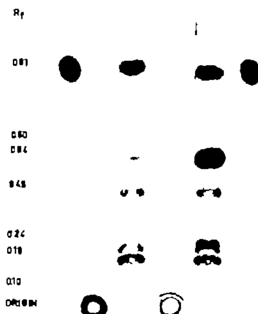


Fig. 3 Autoradiogram of thin-layer chromatogram f chloroform and water phases of urine before and fter hydrolysis with β-glucuronidase. Solvent system ethanol-acetone-benzene-conc. NH_4OH (5 40 50 5) (/v). Solvent front = 16 cm. Exposure time = 7 days. Columns from left to right Standard mepivacaine Water phase before hydrolysis Chloroform phase before hydrolysis, Water phase after hydrolysis Chloroform phase after hydrolysis Standard mepivacaine.

Table 7

Distribution of radioactivity between metabolites found in the chloroform phase and the water phase of urine before and fter hydrolysis with β-glucuronidase. Th values are derived f om the chromatogram shown in fig. 3

	Percentage of total radioactivity	
	before hydrolysis	after hydrolysis
Chloroform phase		
Mepivacaine (R_f = 0.81)	17 7	14 3
R_f = 0.60	0	3.4
R_f = 0.54	2.7	55.4
R_f = 0.45	5.3	5
R_f = 0.24	3 1	6.3
R_f = 0 18	9 4	7.3
R_f = 0.10	0.6	0 8
Origin	1.0	0.1
Water phase		
Origin	60.2	7 1

dase, the radioactivity became soluble in chloroform and was mainly found in the radioactive component with $R_f = 0.54$. The distribution of radioactivity between the chromatographically separated compounds is shown in table 6.

In fig. 3 is shown the autoradiogram of a thin-layer chromatogram of the chloroform and water phases of extracted urine before and after treating the urine with β-glucuronidase. It is the same compound as found in the bile hydrolysate. Quantitative results are shown in table 7.

Discussion

From the results presented here, it can be seen that mepivacaine is rapidly metabolized in the body. Only a small percentage of the drug is excreted into the urine unchanged. The liver is the principal site of mepivacaine metabolism. More than 50% of the administered compound is excreted into the bile as metabolites. The *in vitro* studies showed that liver slices had an appreciable capacity to metabolize the compound and that mepivacaine was mainly metabolized oxidatively. Most of the radioactivity excreted *via* the bile is probably reabsorbed in the intestine and then excreted into the urine, since only a small percentage was found in the faeces.

The nature of the radioactive breakdown products of mepivacaine has been investigated only to a limited extent. N-demethylation plays an important role in the metabolism of the drug, as indicated by the considerable excretion of $^{14}CO_2$ both in intact mice and rat liver slices. In fig. 4 two metabolic routes are suggested. Judged by the $^{14}CO_2$ excretion, demethylmepivacaine could be one of the major metabolic products. This

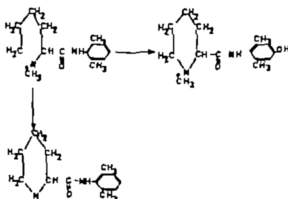


Fig. 4. Routes of metabolism of ^{14}C-mepivacaine in rats.

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Fatal Poisoning in Man by 2,4-Dichlorophenoxyacetic Acid (2,4-D) Determination of the Agent in Forensic Materials

By

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A compound in the group of chlorinated phenoxyacetic acid derivatives, 2,4-dichlorophenoxyacetic acid (2,4-D[1]), herbatox ® herbitox ®) has become most widely used as a chemical herbicide It is stated to be almost non-toxic to animals and man (BUCHER 1946 HILL & CARLISLE 1947 ROWE & HYMAS 1954 DUESBERG & FRIEDERICI 1956 DALGAARD-MIKKELSEN, RASMUSSEN & SIMONSEN 1959 EDSON 1960 DALGAARD-MIKKELSEN & POULSEN 1962 PALMER 1963) However MONARCA & DIVITO (1961) have described a severe case of non-fatal accidental poisoning of a farmer (cf also SEABURY 1963). No account seems to have been given previously of death of a human subject by poisoning by the agent. *In this paper a case is reported of suicide committed by a 23-year-old* farming student[2]) with the dimethylamine salt of 2,4-dichlorophenoxyacetic acid.

The chemical determination of 2,4-dichlorophenoxyacetic acid, which, to our knowledge, has not been undertaken previously on forensic material, *is based on a characteristic absorption curve in the ultraviolet* light after various extraction procedures.

At the time the case was received (1961) no thoroughly tested method was available. For the determinations undertaken a preliminary method was used which in some measure gave too low values, depending on the fatty content of the organs By the method described below more than 80 / of the added substance are recoverable

[1]) 2,4-D (ISO names) is, in our opinion, most inappropriate generic term.
[2]) The reported case of suicide is identical with that briefly mentioned by DALGAARD-MIKKELSEN & POULSEN (1962).

Methods

A. *Determination of 2,4-dichlorophenoxyacetic acid in organs*

To 20 grams minced organ add 5 grams crystalline tartaric acid and 50 ml of chloroform. After homogenisation in a M.S.E. homogenizer transfer the material quantitatively to a 250 ml flask with a glass stopper washing with 50 ml of ether. After adding 20 grams anhydrous Na_2SO_4, shake for 5 minutes. Decant the organic phase, and then extract the residue twice more with 80 ml of ether. Extract the combined organic phase with 40 ml of 0.1 M phosphate buffer after adjusting the pH of the mixture to 7.3. (At this pH and at room temperature the distribution ratio between the phosphate buffer and chloroform is about 75 and that between the phosphate buffer and ether about 17). Repeat the extraction and wash the combined aqueous extract with half its volume of chloroform. Then make the solution strongly acid with 8 N-H_2SO_4, and extract with an equal volume of chloroform, and then with 10 ml. Filter the chloroform extract through anhydrous Na_2SO_4. Extract the filtrate for 5 minutes with 15 ml of 0.02 N borate buffer (pH 10.6). After centrifugation, analyse the clear supernatant spectrophotometrically within the range of 200 to 350 mμ (Beckman DK-2). The curve shows the same absorption maximum at acid and alkaline reaction at 282 mμ, as well as a characteristic point of inflection at 290 mμ.

The concentration is estimated from the difference between the extinction value at 282 mμ and the value resulting at this wave length from intersection by a line passing through the absorption minimum at about 252 mμ and the extinction value at about 310 mμ. By this procedure the concentration is estimated approximately. A more exact value is obtainable by employing WILKIE's (1960) laborious neutralisation method.

B. *Identification of the agent in the specimen received* (*R* 17/61)

a) A sample of the gastric contents was extracted as described and evaporated and sublimed. The residue as a sample of pure 2,4-dichlorophenoxyacetic acid showed, mixed and unmixed, the same (corrected) micromelting point of 138°C.

b) The sublimate mentioned gave the colour reaction recorded by FREED (1948) with chromotropic acid.

c) Spectrophotometric measurement of an aqueous solution of the sublimate yielded the absorption curve characteristic of 2,4-D.

C. *The preliminary extraction method employed in the received case differs in the following respects from that now employed*

Instead of being extracted with phosphate buffer solution, the combined organic phases were filtered through anhydrous Na_2SO_4 and evaporated cautiously to dryness. Then 3 ml of chloroform and 20 ml of 0.1 N H_2SO_4 were added. After heating until the chloroform had been removed, the solution was cooled down to room temperature and filtered. After the whole procedure had been repeated twice, the combined sulphuric-acid extracts were rendered alkaline with 8 N-NaOH and 2 N-Na_2CO_3 to pH about 12. Extraction was performed with equal volumes of chloroform for purification. The filtrates were once more rendered strongly acid with 8 N-H_2SO_4 and then extracted three times with an equal volume of chloroform. The combined dried and filtered chloroform extracts were evaporated. Final volume was 25.0 ml. A me[illegible] red

portion of this was transferred to a 25 ml centrifuge tube with a glass stopper After 10 ml of 0.02 N borate buffer of pH about 11.5 had been added, extraction for 5 minutes and centrifugation, the clear supernatant was analysed spectrophotometrically

D *Recovery experiments using the preliminary and the present extraction methods*

To various amounts of minced human liver (from a normal woman killed in a road accident) 800, 2000 or 4000 µg 2,4-dichlorophenoxyacetic acid were added. Both extraction methods were employed, and the results are set out in table 1 It is seen that between 25 and 65 / of the added quantity was recovered (table 1 left half) by the preliminary method, the larger the amount of tissue the lower the percentage. The bracketed figures represent the relative amounts recoverable in the sulphuric acid residue plus filtered after extraction with 0 1 M phosphate buffer at pH 7.3

By the method described (table 1 right half), most of the added quantities were recovered, independently of the amount of organ analysed.

Table 1

Recovery of 2,4-dichlorophenoxyacetic acid added to human liver estimated by the preliminary as well as the extended extraction method.

By the preliminary method the larger the amount of liver tissue analysed the less agent was recovered. The percentage recovered was independent of the quantity added. The bracketed figures represent the amount found in the sulphuric acid residue plus filter

By the method now used between 80 and 100 per cent were recovered, independently of the amount of liver used.

Amount of liver used g	Preliminary method µg 2,4-D added			Now used method µg 2,4-D added		
	800	2000	4000	800	2000	
5	64.3 (21.3)	63 1 –	– –	99.0	85.1 –	
	61.–	39 6	–	100.1	81 6	Recovery as percentage
10	–	34.8 (46.3)	– –		– –	
20	26.3 (27 0)	26.4 –	27 5 (52.5)	99.0 –	82.2 –	

Case af Fatal Poisoning

A. *Case report*

(L.O 30/61 Forensic-chemical analysis No. R. 17/61 serial no.2279) On Febr 14 1961 at 8.45 a.m. the dead body of a 23-year-old male agricultural student was found in an uninhabited area laying on its back partly undressed. The arms were placed

symmetrically the elbow joints semiflexed the fists were clenched and pressed against the waist. The hands had left numerous imprints on the surrounding ground, where other marks suggested premortal rolling forwards and backwards. Some articles of apparel were found scattered untidily close by Vomit with scant contents of food was seen in two places on the ground and on the clothes of the dead man.

Medico-Legal autopsy undertaken the same day showed the body of a powerfully built 23-year-old man, weighing 75 kg and measuring 178 cm.

There were found pronounced universal rigor and bluish red lividity of medium intensity on the back except on the supporting surfaces. No outward signs of putrefaction were observed. The bodily orifices were found to be normal with no peculiar odour The pupils were of medium size and equal.

Internal examination showed all the organs to be marked by acute congestion. The brain and pia-arachnoid were slightly oedematous. The lungs presented moderate acute emphysema and some hypostasis, but no oedema. The right lung weighed 400 g and the left 310 g. The stomach contained 100 ml of a thin fluid with no peculiar odour and with a neutral reaction. The blood was dark and partly clotted. The liver weighed 1610 g and the spleen 130 g. The whole alimentary tract and all the organs were as stated, congested, but were otherwise normal.

B. *Histological Examination*

1) The central nervous system. Sections from the cerebral cortex, basal ganglia, mesencephalon, hypothalamus, cerebellum, pons and medulla oblongata were stained with haematoxylin-eosin and van Gieson-Hansen s stain, myelin-sheath staining by Weil's method, PAS and Einarson s gallocyanin.

All the sections showed marked congestion with a few perivascular haemorrhages and pericellular oedema. There were found *severe degenerative changes of the ganglion cells* (see fig. 1)

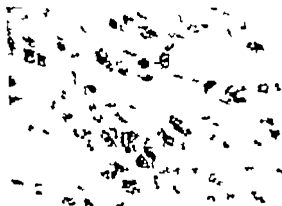


Fig. 1 Degenerating ganglion cells with satellitosis. Pons. Gallocyanin (Einarson) 200.

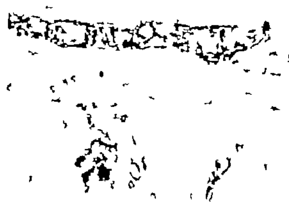


Fig. 2. PAS-positive gran le degenerating ganglion cell nd in capillary wall. Medulla obl. PAS-stain. × 550

Some of these cells were small and shrunken, with intensely stained pyknotic nuclei often situated slightly eccentrically in the scant cytoplasm. The prolongations stood out clearly Other cells were swollen, deformed and clearly chromophobic, with roughly vacuolated cytoplasm and pale nuclei. In some cells there was even a completely eliminated nuclear structure A laminar oedema was seen round the Purkinje s cells, which demonstrated the recorded changes to a pronounced degree. The number of ganglion cells was diminished, especially in the temporal cortex. Pronounced subpial oedema and focal oedematous areas were found everywhere. Marked satellitosis was noticed, especially in the cortex and in the pons (see fig. 1) In a few places round-cell infiltrations were seen perivascularly The vessels were normal. In the PAS-stained preparations there were found PAS-positive granules in numerous degenerate ganglion cells (see fig. 2). Such granules were not seen in the Purkinje s cells. Similar granules were found in several glia cells and round the vessels Early necrosis of the granular layer was seen in the cerebellar cortex, but otherwise no emollitions were noticed The changes were in general most pronounced in the cortex, the globus pallidus, the cerebellum and the pons. The leptomeninges in the insula area presented fresh haemorrhages and excessive oedema.

) Sections from the remaining organs (Staining with haematoxylin-eosin and van Gieson-Hansen s stain lung tissue also for elastin (Weigert) and reticulin (Foot) renal tissue also with PAS the pancreas also with chromalum-haematoxylin-phloxin (Gomori)). *Lungs* Widely distributed spots of acute emphysema with greatly distended alveoli, some of which had fused owing to bursting of the walls. Atelectases were found in interjacent areas. Many of the bronchioles were seen to contain an amorphous, brownish, uncharacteristic, presumably aspirated material The *myocardium* showed normal conditions. The *liver spleen* and *adrenals* presented acute congestion, but no other changes. The *kidneys* displayed pronounced *acute congestion, especially at the site of transition* from the cortex to the medulla. No degenerative changes were observed. The *pancreas* was well-preserved, with no autolysis. Numerous islands of Langerhans were seen, and differential staining showed normal granulation of the beta cells and in places also of the alpha cells. The *prostate* and *testes* were normal.

Sudan-stained frozen sections of the brain, lungs and kidneys showed no signs of fat embolism.

A few days after the postmortem examination a 125 ml bottle was found close to the place of finding the dead body According to the label, this bottle had contained (scant remnants were left) a commercial herbicide consisting of the dimethylamine salt of 2,4-dichlorophenoxyacetic acid at a concentration of 500 g (as the acid) per litre.

C. *Contents of 2,4-dichlorophenoxyacetic acid found in the organs* (*R* 17/61)

The amounts of 2,4-dichlorophenoxyacetic acid found by the preliminary method in various organs are set out in table 2. In the table are given partly the concentrations and partly the total amounts found in the respective organs. As mentioned in the description of the extraction method (see section 1 D) the values must be regarded as minimum values. The recorded total amount of 6 grams may well have represented 50 or even 100 / more.

Though the concentration of the agent in the stomach contents was high, the total amount (about 0 6 g) suggests that the main portion of the

Table 2

Amounts of 2,4-dichlorophenoxyacetic acid in organs (case R 17/61) The values were found by the preliminary method. The total amount found was not less than 6 grams, corresponding to more than 80 mg/kg.

Organ	Weight	Amount of sample	µg 2,4-D per g of organ	mg 2,4-D totally in different organs
Muscles¹)	32.2 kg	20 g	70	2257
Spleen	130 g	20 g	134	
Infarct	–	10 g	134	17
Liver	1610 g	20 g	183	295
Blood²)	4.1 kg	10 ml	669	2740
Kidney³)	270 g	20 g	63	17
Brain	1360 g	40 g	12.5	17
Urine	11 ml	10 ml	264	30
Urine, hydrol.		10 ml	770	–
Contents of				
ventricle	75 ml	2 ml	7770	583
small intest.	65 ml	3 ml	239	16
bowel	80 g	20 g	48	20
Fatty tissue from				
outside of small intest.	?	15 g	129	> 2
outside of bowel	?	15 g	36	> 1
				5995 mg ∾ 6 g

¹) Estimated as 43 % of the body weight (75 kg)
²) Estimated as 5.5 % of the body weight (75 kg)
³) Estimated as 270 g.

amount taken had been absorbed The concentration in the blood was considerably higher than that in the organs. The lowest concentration was found in the brain. The concentration in the urine was about 40 % of that in the blood. The renal excretion must be assumed to have taken place very slowly

The stomach contents had a marked amine like odour (perhaps that of dimethylamine)

The fluid in the bottle proved to contain 560 mg 2,4-dichlorophenoxy acetic acid per millilitre, approximately the concentration stated on the label.

D Other Chemical Analyses Relating to the Case.

The forensic-chemical analyses carried out with a view to establishing the presence of any arsenic, bromine, barbiturates, cyanides, parathion, nicotine, morphine or other alkaloids showed no such substances present in demonstrable amounts.

Discussion

The results of the patho-anatomical examinations and the chemical analyses showed this to be a case of fatal poisoning after oral intake of a concentrated commercial preparation of the dimethylamine salt of 2,4-dichlorophenoxyacetic acid (herbatox D-500 ®) The circumstances related to the place of the corpse, the finding of a bottle containing remnants of the poison mentioned at the commercial concentration, the presence of the poison in all the organs examined and the high concentration in the stomach contents, considered along with information about the psyche of the deceased and the familial occurrence of suicides, suggest that it was a case of completed suicide. Death seems to have been preceded by an interval with convulsions and vomiting.

In the case under review acute congestion of most organs predominated among the patho-anatomical changes. In the brain there were also seen degenerative ganglion-cell changes, consisting of shrunken, degranulated or pyknotic nuclei associated with satellitosis. In the cytoplasm PAS-positive granules were often seen, suggesting accumulation of breakdown products there.

The forensic-chemical analyses for 2,4-dichlorophenoxyacetic acid in the stomach contents, blood, urine and various organs showed its presence in all samples. Next to the stomach contents, the blood was found to have the highest concentration, whereas that in the brain, for instance, was more than 50 times less. Considering that a removed brain probably

contains between 1 and 2 / blood, the true extravascular concentration in the brain may be judged to have been negligible. Provided the cerebral changes recorded were not due to anoxia, we may conclude that the ganglion cells must have been extremely sensitive to the poison.

At the time when the chemical analyses had to be made, only a preliminary method was available, as already stated. The concentrations recorded, especially those in organs with a large fat content, were therefore presumably too low. Unfortunately there was no examination of whether this was also true for brain tissue. In the spleen, the same concentration was found (see table 2) whether extraction was performed from 10 or 20 g tissue, consistent with the low fatty content of this organ, as compared with that of the liver for instance. The total amount of the poison in the body was set at not less than 6 g, corresponding to at least 80 mg/kg. For dogs the LD50 has been found to be 100 mg/kg after oral administration (DRILL & HIRATZKA 1953), whereas for other animals (mice, rats, guinea-pigs and rabbits) it has been found to range between 300 and 700 mg/kg. On the basis of these values it is reasonable to suppose that the minimum amounts found by us could have caused death. The chemical analyses revealed no traces of other poisons.

In experimental animals the poisoning after oral or parenteral administration of the agent (survey see DALGAARD-MIKKELSEN & POULSEN 1962) manifests itself by reduced motor activity with associated rigidity of the skeletal muscles, ataxia and reflex disorders.

In severe cases progressive apathy and muscular weakness are seen, especially in the hindlegs, with associated periodic clonic spasms, loss of weight and final coma. Further states of irritation of the ocular and nasal mucosae are seen, with consequent scratching reactions. Bleedings may occur from the nose and mouth, as well as diarrhoea with blood-stained faeces. Vomiting due to local irritation of the alimentary tract is frequently seen, but may be absent, even after oral administration to dogs. Autopsy of experimental animals has shown necrotic ulcers of the oral mucosa, inflammatory changes with necroses in the small intestine, focal necroses in the liver and degenerative changes of the renal tubules. According to DESI & SHOS (1962), experiments with subcutaneous injection of 200 mg 2,4-D per kilogram to dogs, cats and rats resulted in pronounced changes of the EEG (decreased frequency of the waves and shortening of the desynchronization time) and complete disappearance of conditioned reflexes. These workers took the agent to be accumulated in the brain and recommended measurement of the EEG and testing for conditioned reflexes as an aid to early diagnosis of poisoning with 2,4-dichlorophenoxyacetic acid in man. In our case, however the concentration was found t be lower in the brain than in the other organs. Hence there is reason to suppose that the agent is not especially accumulated in the central nervous system, but that the nervous tissue is highly sensitive to its action.

Only few cases of poisoning with this agent in man have been described previously

In an experiment on himself a worker has taken 500 mg daily for three weeks with no demonstrable effect (ASSOULY 1951) GOLDSTEIN JONES & BROWN (1959) and TODD (1962) have reported cases of protracted polyneuritis in patients exposed to accidental percutaneous absorption of 2,4-dichlorophenoxyacetic acid. MONARCO & DIVITO (1961) described an acute intoxication of a farm worker who for some time had been spraying with the poison one day he inhaled a fairly large amount, because he had the wind against him. Neither the blood or the urine was analysed for the agent. The *main signs and symptoms were of a neurological character* at first ataxia, reflex disorders, such as abolished Achilles tendon reflexes and reduced patellary reflexes, as well as positive Romberg's sign. These phenomena persisted for two or three months and only subsided slowly

About administration to man reference may be made to SEABURY (1963), who tentatively treated two patients suffering from coccidiosis by intra muscular or intravenous injection of the sodium salt of 2,4-dichlorophenoxyacetic acid One of these died of his disease, however after having received no more than 40 mg. The other a man aged 48 received in the course of 34 days a total of 12.7 grams, beginning with very small doses, which were gradually increased. The last two doses were of 2.0 and 3 6 grams. Towards the end of the course of treatment various neurological *signs and symptoms were noted*, reduced reflexes first. There was a transitory improvement, possibly caused by the treatment, but the patient died of his original disease 14 days after the last dose. Histological examination of the brain revealed no changes.

The mechanism of poisoning is as yet unknown A diabetogenic effect through action on the endocrine tissue of the pancreas has been suggested, but this has not been demonstrable in animal experiments (LORENZEN & LYNGSOE 1957) In our case microscopy of the pancreas showed no *abnormality*

Beyond degenerative changes of the cells of the central nervous system (perhaps, however due to anoxia) acute congestion and acute pulmonary emphysema (possibly developed after aspiration of stomach *contents), the* organs presented no abnormalities of any importance.

The risk of fatal accidental poisoning with 2,4-D must be considered generally low In spite of the fairly large dose in the case here reported of suicide by intake of the agent, death is unlikely to have occurred acutely As, *however the changes that may be produced* in the central nervous system after sublethal doses seem to be irreparable, though in mild cases a compensatory reduction to the point of freedom from symptoms may be obtained it is, perhaps, timely to urgently advise *caution in dealing* with and using the chlorated phenoxyacetic acids. Should the skin become

contaminated, the substance should be removed immediately to avoid percutaneous absorption.

The toxicity of the other component of herbatox B 500 ® dimethylamine, is regarded as low (BOVET & BOVET NITTI 1948)

Summary

A man, aged 23 committed suicide by oral intake of the dimethylamine salt of 2,4-dichlorophenoxyacetic acid (commercial herbatox D 500 ®) The lethal dose, in the form of the acid, has been estimated at not less than 6 g, corresponding to at least 80 mg/kg.

Pronounced degenerative changes of the ganglion cells of the brain were found histologically

The contents of the poison in the blood, urine and organs have been recorded The brain had the lowest concentration, less than one-fiftieth of the *blood concentration*.

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Studies on Glucuronide Detoxication Mechanisms in Vitamin A Deficient Rats

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MANVILLE (1937) reported that massive doses of menthol produced gastrointestinal lesions in rabbits resembling those caused by vitamin A deficiency MEUNIER & FERRANDO (1949) showed that the survival and growth of rats given benzoate were directly related to the vitamin A dose. FERRANDO (1950) investigated the effects of vitamin A deficiency on the urinary excretion of hippuric and glucuronic acids by rats given sodium benzoate. They reported that both acids were reduced in the urine of the deficient animals. WEISSMANN (1963) reported the presence of increased β-glucuronidase activity in the serum of guinea pigs given high doses of vitamin A.

The above mentioned studies indicated that vitamin A might play a role in the glucuronide detoxication mechanisms. It was therefore considered useful to investigate the effect of vitamin A deficiency on the enzyme systems that carry out the conjugation and hydrolysis of glucuronides.

In the study reported here we investigated the effect of vitamin A deficiency on the detoxication of benzoate, menthol and phenolphthalein, on the activity of β-glucuronidase in several organs and on the activity of uridine diphosphate transglucuronylase in rat liver slices.

Methods

Weanling albino rats from our own colony were kept individually in wire-bottomed cages in a temperature-controlled laboratory Each experiment involved t[illegible] of littermates Vitamin A-deficient, pair-fed controls and ad libitum fed [illegible] h. The rats received a vitamin A-deficient diet of the composition casein 15, [illegible] ur

(45 % extraction) 68, dried brewer's yeast 8, arachis oil 5 and salt mixture (SURE 1941) 4 %. Twice weekly each vitamin A deficient rat was given by dropper 100 I.U. vitamin D_3 and 0.2 mg D-α-tocopherol dissolved in arachis oil. Each control animal in addition received 1000 I.U. vitamin A palmitate in its supplement. The animals were considered deficient when cessation of growth was observed. At this stage vitamin A could not be found in the liver. The rats were bled, and the organs were quickly removed and chilled. The various assays were carried out immediately.

Detoxication experiments were carried out by administering the different compounds by stomach tube. The dosage per rat in the various experiments were: 100 mg menthol dissolved in 1 ml arachis oil, 10 mg phenolphthalein dissolved in 1 ml of arachis oil and 100 mg sodium benzoate dissolved in 2 ml dist. water. The rats were placed in metabolic cages with all glass receptacles for separating urine and faeces. The urine was collected for 24 hours after dosing. The total glucuronic acid excreted in the urine was determined by the method of HANSON *et al.* (1944).

β-Glucuronidase was determined as described by PELLEGRINO & VILLANI (1956). The organs to be examined were quickly weighed and homogenized in chilled distilled water in a POTTER ELVEHJEM glass homogenizer fitted with a "teflon" pestle. All tissues were homogenized for one minute at a speed of 1250 RPM. The homogenates were diluted to a final concentration of 5 mg wet tissue per ml. Phenolphthalein mono-β-glucuronic acid (Sigma Chem. Co.) was used as substrate. Incubation was for one hour at 38° in 0.2 M acetate buffer at pH 5.2. The total enzyme activity was expressed as mg phenolphthalein liberated per g wet tissue per hour.

Uridine diphosphate transglucuronylase was determined in liver slices as described by DUTTON & STOREY (1962). Tissue slices from chilled livers (c. 10 mg dry weight) were incubated for 90 minutes at 37° in a DUBNOFF metabolic shaker (60 strokes/min.) in an atmosphere of 5 % CO_2 in O_2. o-Aminophenol (Sigma Chem. Co.) was used as substrate. The o-aminophenyl glucuronide formed was measured spectrophotometrically after diazotization and coupling to N-1-naphtylethylenediamine. The enzyme activity was expressed as mg o-aminophenyl glucuronide formed per g dry tissue per hour.

Results

The detoxication experiments are summarized in table 1. The results show that, in the absence of stress compounds, the urinary excretion of glucuronic acid was similar in all experimental groups. In the deficient as well as the control rats, sodium benzoate and menthol in doses of 100 mg per rat resulted in an increased excretion of urinary glucuronic acid, whereas 10 mg phenolphthalein per rat appeared to decrease it. When the deficient and the pair fed groups are compared, the results indicate a lower excretion of urinary glucuronic acid in the deficient group after dosing with menthol or phenolphthalein but not with benzoate.

The β-glucuronidase activities found in the organs of the rats are summarized in table 2. As distilled water was used for preparing the tissue homogenates, the values obtained represent the "total" enzyme in the tissue (GIANETTO & DE DUVE 1955). The results show that the enzyme

Table 1

Urinary excretion of glucuronic acid after administration of toxic compounds to vitamin A-deficient and normal rats.

Compound administered	Dose mg per rat	Mg glucuronic acid excreted in 24 hrs. Vitamin A deficient (5)[1]	Control groups Pair fed (5)	Ad libitum fed (5)
None	–	27 7 ± 1.0[2])	28.6 ± 1.8	30.4 ± 1 4
Sodium Benzoate.	100	60.0 ± 3 3	53.5 ± 2.2	50 0 ± 3.2
Menthol	100	38.1 ± 3.0	46.1 ± 1.8	35.8 ± 3.0
Phenolphthalein	10	18 7 ± 2 0	25 0 ± 4.0	13.0 ± 2.4
(Mean body weights g)		(140)	(161)	(201)

[1]) No. of rats in the group.
[2]) ± standard error of the mean.

activity was higher in the livers of the vitamin A-deficient rats than in those of the pair-fed or full fed controls. This difference was statistically significant ($p < 0.05$) The enzyme activities of the spleen, kidneys and intestine were not affected by the vitamin A deficiency The β-glucuronidase activity per g tissue was considerably higher in the liver and spleen than in the kidneys and intestines.

The UDP transglucuronylase activities are reported in table 3 The use of the liver-slice technique in the assay introduces a number of variables, so that the values are not strictly comparable. The results obtained, how

Table 2

β-Glucuronidase activity in tissues of vitamin A-deficient and normal rats. The values are reported as mean ± standard error The small intestine refers to the section from the stomach to the caecum, the large intestine from the caecum to the anus. Both were rinsed free of contents.

Organ	mg phenolphthalein liberated per g wet tissue/hour Vitamin A deficient (6)[1])	Control groups Pair fed (6)	Ad libitum fed (6)
Liver	36 0 ± 6.3[2])	29 2 ± 4.6	25.9 ± 5.2
Spleen	37 0 ± 4 4	35.5 ± 3.2	35.2 ± 6 6
Kidney	6 4 ± 0 7	6.9 ± 0 7	7.0 ± 0.6
Small intestine	5 2 ± 0.4	5.4 ± 0 5	4.8 ± 0.4
Large intestine	6.4 ± 0.6	6.9 ± 0.8	6 8 ± 1.2

[1]) No. of rats in the group.
[2]) In extended studies with groups of 8 rats the difference between liver values from vitamin A deficient and pair-fed controls was highly significant ($p < 0.001$).

Table 3

UDP-transglucuronylase in liver slices of vitamin A-deficient and normal rats.

Group	Number of rats	Mean weights g		Mg o-aminophenyl glucuronide formed per g dry tissue/hour	
		Body	Liver	Mean	Range
A-deficient	8	150	4.75	0.122	0–0.524
Pair fed controls	8	158	4.92	0.129	0–0.409
Ad libitum fed controls	8	195	7.48	0.091	0–0.257

ever indicated that the glucuronide-conjugation activity of rat liver was not affected by vitamin A-deficiency. There was a wide spread of values within each experimental group but no consistent tendency in the observed enzyme activities was noted. The somewhat lower activity per g dry weight in the full-fed controls was not related to the vitamin A status, but probably due to the full-fed having larger livers than the pair fed rats.

Discussion

The importance of the liver in detoxication is well recognized impairment of this process in vitamin A-deficient rabbits was reported by MANVILLE (1937). Our experiments (table 1) showed that the basal excretion of glucuronic acid in the urine was not affected by vitamin A-deficiency. This indicated that the biosynthesis of glucuronic acid did not require vitamin A, in agreement with the conclusion drawn by WOLF & VARANDANI (1960) from their isotope studies.

FERRANDO (1950) investigated the urinary components of rats fed 2 % sodium benzoate in the diet and found a greatly decreased excretion of glucuronic acid in the vitamin A-deficient rats compared with controls given vitamin A. This finding suggested the possibility that vitamin A may be required in a specific enzymatic reaction involving glucuronic acid. Our findings (table 1) do not agree with those of FERRANDO (1950) but clearly show that the administration of benzoate or menthol caused an increase in the urinary excretion of glucuronic acid in both the deficient and the control group.

The results presented indicate a possible mechanism by which gastrointestinal lesions resembling those of vitamin A deficiency were induced in rabbits and dogs given compounds which require glucuronic acid for

detoxication (MANVILLE 1937 and HARTIALA 1955) It is possible that the increased excretion of glucuronic acid involved in detoxication may reduce the available supply for the biosynthesis of mucopolysaccharides. WOLF & VARANDANI (1960) reported that mucopolysaccharide-biosynthesis require vitamin A.

The effect of vitamin A-deficiency on the detoxication of the compounds studied was not pronounced and did not follow a consistent pattern. More detailed studies, in which the total effect of each toxic compound is considered, are required before any definitive conclusions can be drawn.

The UDP transglucuronylase activity of rat liver slices was not affected by vitamin A-deficiency under the conditions employed in our study (table 2). Thus it may be concluded that the synthesis of glucuronides in the liver did not require vitamin A. On the other hand, the results for β-glucuronidase (table 3) show a significant increase in the activity of this enzyme in liver homogenates of vitamin A-deficient rats. In view of the reports of DINGLE (1961) and FELL (1962) on the effect of vitamin A on lysosomal membranes, it is probable that the observed increase in β-glucuronidase activity in vitamin A-deficient rat liver homogenates was due to an indirect effect of the vitamin on these membranes This assumption is further supported by the finding reported by WEISMANN (1963), who found an increase in the activity of another lysosomal enzyme, namely sulphatase, in the serum of guinea pigs during hypervitaminosis A.

Summary

The effect of oral doses of sodium benzoate, menthol or phenolphthalein on urinary glucuronic acid excretion in vitamin A-deficient as well as in pair-fed and in ad-libitum fed control rats was studied. In all experimental groups a 100 mg dose of sodium benzoate or menthol increased glucuronic acid excretion, whereas a 10 mg dose of phenolphthalein appeared to cause a decrease. Vitamin A-deficiency was without effect on the basal urinary glucuronic acid excretion, and the effect of the deficiency on the excretion of the acid after the administration of the different compounds was neither pronounced nor consistent.

Liver homogenates from vitamin A-deficient rats showed higher β-glucuronidase activity than pair-fed and full-fed controls. No similar effect was observed in the spleen, kidneys or intestines.

The ability of liver slices to conjugate o-aminophenol with glucuronic acid, as measured by UDP transglucuronylase assays, was not affected by vitamin A-deficiency

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The Effect of Nalorphine on Morphine and Codeine Analgesia and Lethality and the Interaction of Morphine and Codeine

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In previous studies we have compared the analgesic effect of morphine and codeine and the conversion of codeine to morphine in tolerant and non-tolerant rats (Jóhannesson & Schou 1963 Jóhannesson & Woods 1964 Jóhannesson, Rogers, Fouts & Woods 1964) In the investigation reported here, comparison has been made between the antagonistic effect of nalorphine on the analgesic actions of morphine and codeine It has recently been shown that simultaneous intraperitoneal injections of large amounts of morphine and nalorphine to rats can result in brain concentrations of nalorphine significantly higher than those found after nalorphine given alone (Jóhannesson & Milthers 1963 Jóhannesson 1963). The biological implication of these results could be that intraperitoneal nalorphine is a more potent antagonist than subcutaneous to the analgesic actions of morphine and codeine In the experiments recorded here, the drugs were therefore administered to rats by both routes.

Jóhannesson & Woods (1964) found that in the rat subcutaneous morphine was a more potent analgesic than intraperitoneal morphine, whereas for codeine the reverse was true. In order to determine whether the same pattern is characteristic of the lethal effects, morphine and codeine were administered by subcutaneous and intraperitoneal injections and the lethalities recorded. It was also investigated whether nalorphine exerted any antagonistic effect on the lethal action of morphine and codeine in the rat.

The interaction of morphine and codeine has apparently not been extensively studied in man or in animals (cf Krueger, Eddy & Sumwalt 1943). However the results of Rikl (1928) indicated that codeine may exert an antagonistic as well as an additive effect on the

respiratory depression caused by morphine. Morphine and codeine were therefore administered by subcutaneous injections to rats, and comparison were made of the lethal effect of the drugs given separately and simultaneously. Experiments were also conducted to evaluate whether codeine has an antagonistic or an additive effect on morphine analgesia.

Methods

Animals. Male Holtzman rats (180–300 g), maintained in constant environmental conditions with free access to water and a commercial food preparation, were used. The studies described were conducted during May, June, July and August 1964 at a room temperature varying from 22–26°.

Injections. Morphine sulphate, codeine phosphate or nalorphine chloride was dissolved in water and given by subcutaneous or intraperitoneal injections. In analgesia experiments drugs were given by tuberculin syringes, adjusted to deliver 0.01 ml, in amounts of 2 ml/kg. In toxicity experiments injected solutions were administered in amounts of 6 ml/kg. The animals therefore received twice these volumes when two drugs were given.

Intraperitoneal injections were given in the middle of the abdomen at the midline. Subcutaneous injections were given in the middle of the back at the midline or in the gluteal region. Subcutaneous injections of morphine were always given in the middle of the back, whereas nalorphine was always given in the gluteal region. Codeine was given subcutaneously in the back, like morphine, except when morphine plus codeine were given to the animals, then codeine was injected in the gluteal region. When morphine and codeine, or morphine and nalorphine, were given to the same animal, morphine was injected first, and then the other drug immediately from another syringe. When codeine and nalorphine were given to the same animals, codeine was given first and then nalorphine immediately. The doses quoted refer to the drugs as free bases.

Experiments on the degree of analgesia. Subcutaneous injections of 5.0 mg/kg of morphine or 60 mg/kg of codeine and intraperitoneal administration of 10.0 mg/kg of morphine or 30 mg/kg of codeine result in a high and equal degree (assessed statistically) of analgesia in the rats 30 minutes after the injections (cf. JÓHANNESSON & WOODS 1964). In order to evaluate the influence of nalorphine on the analgesic action of morphine and codeine, it was administered by subcutaneous or intraperitoneal injections simultaneously with morphine or codeine in the doses mentioned above. Nalorphine was given in amounts of 5.0, 2.5, 1.25, 0.63 or 0.032 mg/kg by subcutaneous and 10.0, 5.0, 2.5, 1.25 or 0.63 mg/kg by intraperitoneal injections. It was further investigated whether high doses of nalorphine (5–50 mg/kg) exerted any analgesic effect on the rats.

In testing the influence of codeine on morphine analgesia, various doses of codeine were administered simultaneously with 5.0 or 2.5 mg/kg of morphine. Both drugs were given subcutaneously. Codeine was administered in amounts of 60.0, 30.0, 15.0, 7.5 and 3.5 mg/kg. For comparison, these doses of codeine alone were given to several rats.

The degree of analgesia was determined 30 minutes after the injections by the hot plate method of EDDY *et al.* (1950, 1953) as modified by JÓHANNESSON & WOODS (1964). Except for experiments in which nalorphine was given alone, the groups of rats used for each dose numbered about 26 animals, each animal being used twice, with a week's

interval. The results are based on determinations on almost equal (equal ± 1) numbers of rats used for the first and the second time. These experiments were performed and completed before it was realized that even 3 weekly injections of an analgesic test dose of morphine can induce tolerance to some extent in the rats (JÓHANNESSON & WOODS, unpublished results).

Toxicity experiments. Our interest was primarily to determine the acute toxicity of morphine and codeine in rats. On the basis of an earlier investigation into the acute toxicity of intraperitoneal morphine for rats, a survival period of 60 minutes was considered appropriate for the present purpose (cf. JÓHANNESSON 1962). The toxic effects of morphine and codeine given by intraperitoneal injections were first compared. Many of the rats died within the first 60 minutes: the rats then alive were taken as survivors. However, when similar or greater amounts of the drugs were administered subcutaneously, only a few animals died within the first 60 minutes. The survival period was therefore extended to 120 minutes (morphine) or 180 minutes (codeine) when the drugs were administered subcutaneously.

We attempted to evaluate the minimal lethal doses as well as the doses that were lethal to almost all the animals. For this purpose doses of a sufficiently wide range were administered to the animals by subcutaneous or intraperitoneal injections. Each dose was tested on a group of 10 animals. Respiratory arrest was taken as the sign of death.

Morphine was given in amounts of 15, 30, 50, 75, 100, 125, 200, 300 and 350 mg/kg intraperitoneally. The last dose was a supersaturated solution of morphine sulphate and had to be given lukewarm. Except for the omission of this dose, the same doses were, with two minor alterations (150 mg/kg instead of 125 g/kg and an additional dose of 250 mg/kg), administered subcutaneously. Codeine was given intraperitoneally in amounts of 70, 100, 125, 150 and 180 mg/kg and 100, 200, 250, 300, 400, 500, 600 and 700 mg/kg subcutaneously.

The effects of nalorphine on the lethal actions of morphine and codeine were investigated. For this purpose 100 or 200 mg/kg of nalorphine were given subcutaneously or intraperitoneally with morphine or codeine in amounts expected to kill 50% and 70% of the animals. These amounts of nalorphine given alone had no lethal effect in our experiments. In order to evaluate the interaction of morphine and codeine on the lethal action in the rats, morphine and codeine were administered simultaneously by subcutaneous or intraperitoneal injection in amounts expected to kill 50% and 30% of the animals. Morphine was also given in the 30% lethal doses with doses of codeine too low to kill any animals at all if given alone.

Subcutaneous or intraperitoneal injections of 12.15 ml/kg of the water used for making the drug solutions had apparently no adverse effect on the animals. All the animals tested survived for at least 24 hours.

In tests of significance, the null hypothesis was rejected if $P < 0.05$. The χ^2 test was performed as described by BURN *et al.* (1950) and the "t" test by SAUNDERS & FLEMING (1957).

Results

A. *Degree of analgesia.*

1. Analgesic effect of nalorphine. In doses lower than 10 mg/kg nalorphine had no measurable analgesic effect 30 minutes after the injections. However, when higher doses (15, 30 and 50 mg/kg) were given, a few animals were found under analgesia, but dose-response curves could not be established.

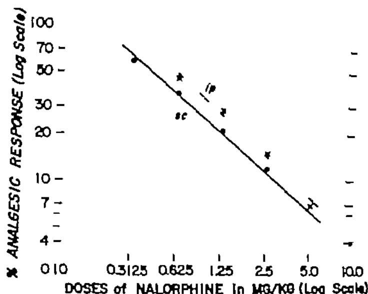


Fig. 1 The effect of nalorphine on the analgesic effect of codeine in tests performed 30 minutes after the injections. The drugs were given simultaneously by subcutaneous (s.c.) or intraperitoneal (i.p.) injections, 25–29 animals being used for each dose.
The abscissa is the dose of nalorphine in mg/kg (log scale).
The ordinate is the percentage of animals showing positive analgesic responses (log scale).
Codeine was always given in amounts of 60 mg/kg by subcutaneous and 30 mg/kg by intraperitoneal injection. Given alone to 29 and 26 rats, these doses produced analgesia in 76% and 77% of the animals 30 minutes after the injection.

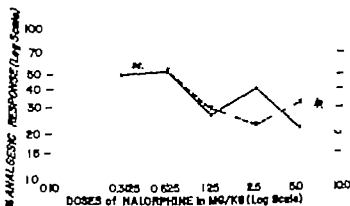


Fig. 2 The effect of nalorphine on the analgesic effect of morphine in tests performed 30 minutes after the injections. For explanation, see fig. 1.
Morphine was always given in amounts of 5.0 mg/kg by subcutaneous and 10.0 mg/kg by intraperitoneal injections. Given alone to 28 and 27 rats, these doses produced analgesia in 71% and 70% of the animals 30 minutes after the injections.

2. **Effect of nalorphine on codeine and morphine analgesia.** Nalorphine was a potent antagonist to the analgesic effect of codeine, whether the drugs were given subcutaneously or intraperitoneally (fig. 1) The results show that only 12–15 / of the animals were under analgesia after injections of 2.5 mg/kg of nalorphine plus codeine (60 mg/kg sc. or 30 mg/kg ip), whereas the injections of codeine alone would have resulted in a degree of analgesia 5–6 times higher The results indicate further that nalorphine was more potent subcutaneously than when given intraperitoneally It is noted that the degree of analgesia observed after 5 mg/kg of nalorphine plus the codeine given subcutaneously is obviously askew There were 27 animals in this group and of them 3 were under analgesia. One of those was probably a false positive reactor

In contrast to the results of experiments with nalorphine and codeine, the antagonistic effect of nalorphine on morphine analgesia did not increase regularly with increasing doses (fig. 2) Thus, intraperitoneal injections of 5 mg/kg of nalorphine had less antagonistic action than 2.5 mg/kg and approximately the same effect as 1.25 mg/kg. When nalorphine was given subcutaneously 2.5 mg/kg exerted an apparently but not significantly weaker (χ^2 test) antagonistic effect on morphine analgesia than was observed after 5 0 or 1 25 mg/kg.

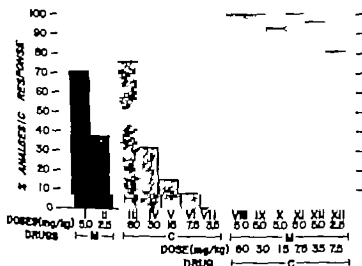


Fig. 1. The analgesic effects of morphine, codeine and morphine plus codeine in rats. The drugs were given subcutaneously and simultaneously if both drugs were administered t the animals. The degree of analgesia was determined 30 minutes later and expressed as the percentages of animals showing analgesia. The number of animals used in each group was 25–29

M Morphine C Codeine. Columns I and II refer to experiments with morphine columns III–VII refer to experiments with codeine columns VIII XIII refer to experiments I both drugs were given.

Morphine and codeine were administered in equi-analgesic doses (cf figs. 1,2) However high doses of nalorphine (1 25 mg/kg and higher) exerted a greater antagonistic effect on codeine than on morphine analgesia (figs. 1,2)

3 Morphine plus codeine on the degree of analgesia (fig. 3). Simultaneous subcutaneous injections of morphine and codeine did not result in an antagonistic effect on the degree of analgesia in the rats (cf columns I–VII and VIII–XIII) Thirty eight and 71 / of the animals were under analgesia after they had been given 2.5 and 5 0 mg/kg of morphine alone (columns I and II) No analgesic effect was observed after 3 5 mg/kg of codeine and only a low degree of analgesia (8 /o) after 7.5 mg/kg (columns VI and VII). When 5 0 mg/kg of morphine had been given with these amounts of codeine, all but one of the animals tested were under analgesia (columns XI and XII) Simultaneous administration of 2.5 mg/kg of morphine and 7.5 mg/kg of codeine gave a degree of analgesia (81 /o) (column XIII) high enough to be significantly greater (χ^2 = 22.5) than the summed analgesic effect of these doses of morphine and codeine given separately

B *Lethality*

1 Codeine lethality Intraperitoneal codeine was approximately four times as toxic as subcutaneous codeine (fig. 4) Further the rats lived on an average longer after subcutaneous than after intraperitoneal codeine.No rat died within the first 60 minutes after subcutaneous codeine. The mean time of death occurring within the first 60 minutes after intraperitoneal codeine was thus significantly different ("t" test) from the mean time till death within the first 180 minutes after subcutaneous codeine.

Death after codeine was invariably associated with generalized convulsions. One hundred, 200 and 250 mg/kg given subcutaneously or 70 mg/kg given intraperitoneally had no lethal effect, although some of the rats developed convulsions.

2 Morphine lethality Administration of high as well as of low doses could result in a lethal effect. The results of experiments in which doses higher than 100 mg/kg were given to the rats are plotted in fig. 4 Death occurred later after subcutaneous than after intraperitoneal morphine The dose-response curves thus refer to the numbers of animals dying within 60 and 120 minutes, respectively When comparison is made at these levels, the relative potency is almost 1 1 However the mean times of death after subcutaneous and intraperitoneal morphine were, as found for codeine, statistically different ("t" test). Most of the rats that

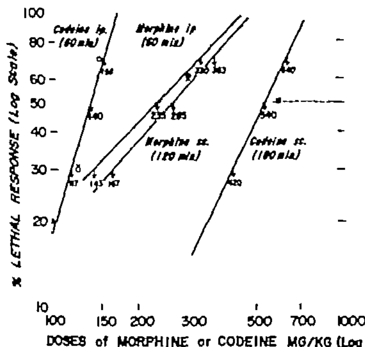


Fig. 4. The lethal effects of morphine (high doses) and codeine in rats. The drugs were given subcutaneously (s.c.) or intraperitoneally (i.p.), 10 animals being used for each dose. Figures in brackets denote that the rats were taken as survivors if they survived to 60 minutes after intraperitoneal morphine or codeine, for 120 minutes after subcutaneous morphine or for 180 minutes after subcutaneous codeine (see text).
The abscissa is the dose administered in mg/kg (log scale).
The ordinate is the percentage of animals dying within the periods defined for each of the four experiments (log scale). The arrows on the abscissa indicate the doses of morphine and codeine that were expected to kill 30 %, 50 % or 70 % of the animals (LD30, 50 and 70).

survived 60 minutes after intraperitoneal morphine were, moreover dead at 120 minutes after

The results of experiments in which 100 mg/kg or lower amounts were given are shown in table 1 Few animals died within the first 60 minutes after the lower doses of morphine, whether subcutaneous or intraperitoneal. Comparison was therefore made at 120 minutes. The lethal effect of subcutaneous morphine did not follow a regular pattern. Thus, subcutaneous morphine in amounts of 30 100 or 250 mg/kg was found to kill 5 out of 10 animals (cf. fig. 4 and table 1)

Death after morphine occurred in flaccid respiratory paralysis, whether high or low doses had been given.

At the LD50 level, intraperitoneal codeine was 1 7 times as potent as intraperitoneal morphine.

Table 1

The lethal effect of *morphine (low doses) on the rat.* Morphine was given subcutaneously or intraperitoneally in amounts of 100 mg/kg or less to rats in 10 groups, 10 animals in each. The numbers of rats in each group that died within 120 minutes after the injections and those of rats that survived this period are shown. The numbers in brackets denote rats dying within the first 60 minutes after the injections.

	Subcutaneous morphine		Intraperitoneal morphine	
Doses mg/kg	Nr of animals dying	Nr of animals surviving	Nr of animals dying	Nr of animals surviving
15	2 (0)	8	0	10
30	5 (1)	5	1 (1)	9
50	3 (1)	7	3 (1)	7
75	7 (0)	3	5 (0)	5
100	5 (0)	5	4 (0)	6

2. Nalorphine on morphine and codeine lethality (table 2). Nalorphine alone as previously mentioned, had no acute lethal effect in the doses used (100 or 200 mg/kg). Nalorphine could completely antagonize the lethal effect of morphine for 24 hours at least. No rat given morphine and nalorphine subcutaneously died. A complete antagonism of morphine lethality was observed when the lower dose of nalorphine

Table 2

The effects of nalorphine on morphine and codeine *lethality in rats. The drugs were,* with two exceptions, given simultaneously by subcutaneous or intraperitoneal injections to rats in 7 groups, 10 animals in each. The expected percentage mortalities after morphine or codeine given alone are shown in column 5 (cf Fig. 4).

Drugs given	Doses mg/kg	Nr of animals surviving[2])	Nr of animals dying[3])	LD (%) of morphine or codeine alone
Codeine + Nalorphine *i.p.*	140 + 100	1	9	50
Morphine + Nalorphine *i.p*	235 + 100	10	0	50
Morphine + Nalorphine *s.c* [1])	265 + 100	10	0	50
Morphine + Nalorphine *i.p*	235 + 200	5	5	50
Morphine + Nalorphine *s.c* [1])	265 + 200	10	0	50
Morphine + Nalorphine *s.c*	265 + 200	10	0	50
Morphine + Nalorphine *i.p*	330 + 100	7	3	70

s.c injected subcutaneously
i.p injected intraperitoneally
1) Nalorphine given 30 min. after morphine.
2) With a few exceptions, the animals survived for 24 hours at least.
3) Numbers of animals dying within 60 min. after morphine plus nalorphine *i. p.* or codeine plus nalorphine *i.p* or within 120 min. after morphine plus nalorphine

(100 mg/kg) was given with the LD50 of morphine intraperitoneally However no antagonism could be demonstrated when the higher dose of nalorphine (200 mg/kg) was given with the morphine Intraperitoneal administration of the lower dose of nalorphine and the LD70 of morphine resulted in partial antagonism of the lethal effect. Death after simultaneous administration of morphine and nalorphine was preceded by more or less generalized convulsions.

Nalorphine increased rather than decreased the lethal effect of codeine. The rats also apparently died earlier after codeine plus nalorphine than after codeine given alone (all 9 rats died within 25 minutes), but the difference was not statistically significant. Death after codeine plus nalorphine occurred in generalized convulsions.

4 The lethal effect of morphine plus codeine. The results are summarized in table 3 They show that morphine and codeine injected simultaneously potentiate one another s lethality for rats. When the LD30 of morphine was given subcutaneously or intraperitoneally with codeine in amounts too low to exert any lethal effect of their own, 8 and 10 out of 10 rats died. Nineteen out of 20 rats injected subcutaneously with morphine plus codeine died within 180 minutes of the injections. All but 3 of these rats died 55 to 120 minutes later Their mean time of death was thus appreciably but not significantly lower than that of rats dying after subcutaneous codeine only Death after morphine plus codeine was usually preceded by generalized convulsions.

Table 3

The lethal effects of morphine and codeine in rats. The drugs were given simultaneously by subcutaneous or intraperitoneal injections to rats in 5 groups, 10 animals in each. The expected percentage mortalities after morphine and codeine given alone are shown in columns 4 and 5 (cf Fig. 4)

Doses mg/kg	N of animals surviving	Nr of animals dying[1])	LD (%) of morphine alone	LD (%) of codeine alone
235 (M) + 140 (C) i.p.	0	10	50	50
143 (M) + 117 (C) i.p.	0	10	30	30
143 (M) + 70 (C) i.p.	2	8	30	0[2])
167 (M) + 420 (C) s.c.	1	9	30	30
167 (M) + 250 (C) s.c.	0	10	30	0[2])

(M) Morphine, (C) Codeine.
s.c. Injected subcutaneously
i.p. Injected intraperitoneally
[1]) Numbers of animals dying within 60 minutes after morphine plus codeine i.p. or within 180 minutes after morphine plus codeine s.c.
[2]) See text.

Discussion and Conclusions

The results suggest that nalorphine may exert a more or less biphasic effect on morphine analgesia (fig. 2). In this connection the results of RUBIN, CHERNOV MILLER & MANNERING (1964) must be mentioned. They found in experiments on rats, using the tail flick method for testing analgesia, that the maximum antagonism of nalorphine (among certain other narcotic antagonists) on morphine analgesia was reached as the ratio morphine/nalorphine was decreased, but that after a certain point the effect decreased with decreasing ratios. WINTER *et al* (1954) and HARRIS & PIERSON (1964) have, on the other hand found that the antagonistic effect of nalorphine on morphine analgesia in the rat is regular and strictly related to dose when the tail flick method is used. Results therefore are highly contradictory

In our experiments nalorphine was found to be a more potent antagonist to the analgesic action of codeine than to that of morphine. Nalorphine also antagonized morphine and codeine analgesia differently in so far as increasing amounts of nalorphine always resulted in increased antagonism to the analgesic effect of codeine (fig. 1) It is therefore concluded that the analgesic actions of codeine and of morphine are essentially different, whether the drugs are given subcutaneously or intraperitoneally (figs. 1 2).

Death after codeine in the rat was always associated with generalized convulsions in these experiments. Death after morphine always occurred in flaccid respiratory paralysis without any preceding convulsions. The lethal actions of morphine and codeine are therefore probable due to different mechanisms of action. This view is supported by the experimental fact that nalorphine, which increases rather than decreases the lethal action of codeine, is usually a strong antagonist to the lethal action of morphine (table 2)

The LD50 of intraperitoneal codeine was about 4 times lower than that of subcutaneous codeine (fig. 4) Even so death occurred significantly later after subcutaneous codeine (see results) JÓHANNESSON & WOODS (1964) found that intraperitoneal codeine is about twice as potent an analgesic as subcutaneous codeine On the basis of plasma concentrations in rats given codeine subcutaneously or intraperitoneally they concluded that significantly greater amounts of morphine were formed from codeine *in vivo* when it was given intraperitoneally The question therefore arises whether the enhanced biological activity of intraperitoneal codeine is primarily due to greater amounts of biotransformed morphine The results of the experiments with nalorphine on the analgesic and toxic actions of morphine and codeine are not compatible with the idea that biotransformed morphine plays a major part in the biological activity of codeine

Although biotransformed morphine has been found in the brain of rats after low doses of codeine (JÓHANNESSON & SCHOU 1963), it is nevertheless conjectural at the present time whether it can be assigned a biological function or not. Other phenomena, such as rates of absorption, must accordingly be taken into consideration in order to explain the greater activity of intraperitoneal codeine (cf JÓHANNESSON & WOODS 1964) However simultaneous injections of morphine and codeine resulted in a potentiating effect on the degree of analgesia and lethality in the rats (fig. 3 table 3). In conclusion it can therefore be said that possibly morphine, *biotransformed from codeine* does not interact with codeine in the brain of rats in the same way as does morphine *injected along with* codeine.

The potentiation of injected morphine and codeine deserves much more attention, e.g. in clinical pharmacologic studies on the human subject. It may be mentioned here that codeine, in doses too low to exert any analgesic effect of their own, can significantly increase the intensity and persistence of morphine analgesia in rats (JÓHANNESSON & WOODS, unpublished results) It should also be noted that rats given both morphine and codeine die in generalized convulsions, as if they had received codeine alone. Apparently morphine can somehow increase the lethal and convulsant action of codeine.

Given in high doses, nalorphine had a low and irregular analgesic effect of its own. Nalorphine can be transformed to normorphine *in vivo* in the rat (MILTHERS 1962 JÓHANNESSON & MILTHERS 1963) and normorphine is a weak analgesic in rats (JÓHANNESSON & SCHOU 1963). The sporadic analgesic action of nalorphine can therefore be associated with its conversion to normorphine.

JÓHANNESSON & MILTHERS (1963) have demonstrated that high but non-lethal doses of morphine and nalorphine can cause death in rats if given simultaneously by the intraperitoneal route. This potentiation could probably be explained by the fact that the simultaneous injections of the drugs resulted in a brain concentration of nalorphine significantly higher than that found when it had been given alone (JÓHANNESSON & MILTHERS 1963 JÓHANNESSON 1963) In the experiments recorded here (table 2), nalorphine given subcutaneously constantly and completely antagonized the lethal action of subcutaneous morphine (LD50) However when the drugs were given intraperitoneally only the lower dose of nalorphine (100 mg/kg) was found to antagonize morphine lethality (LD50), and no effect was observed after the higher dose (200 mg/kg) The missing antagonism of the higher dose of nalorphine given intraperitoneally may be associated with the high amounts of nalorphine found in the brain after simultaneous intraperitoneal injections of large amounts of the two drugs.

In conclusion, it can therefore be said that the antagonism of nalorphine to morphine lethality in the rat can depend on the route of administration, the dose injected and probably the brain concentration of the antagonist.

In doses *higher* than 100 mg/kg, intraperitoneal morphine was found more toxic than subcutaneous morphine. Thus, almost the same percentage of animals died within the first 60 minutes after intraperitoneal morphine as within 60–120 minutes after subcutaneous morphine (fig. 4). At *lower* doses (50, 75 and 100 mg/kg) the lethal effect was approximately the same, whether the morphine had been given subcutaneously or intraperitoneally (table 1). At the *lowest* doses given (15 and 30 mg/kg), subcutaneous morphine was, however, more toxic than intraperitoneal morphine (table 1). These last mentioned results are consistent with the fact that morphine (in still lower doses) is approximately a twice more potent analgesic when given by subcutaneous than by intraperitoneal injections (JÓHANNESSON & WOODS 1964). The available experimental results therefore indicate that the ratio of the biological effects of subcutaneous *versus* intraperitoneal morphine is greatest at the lowest doses. The investigation reported here offers no ready explanation of these phenomena. However, a possible explanation could be that the rate of intraperitoneal absorption increases relatively as the concentration of injected morphine is increased, whereas subcutaneous absorption does not. It is relevant that subcutaneous morphine has been found to depress its own rate of absorption owing to release of histamine and 5-hydroxytryptamine (SCHOU 1961). Whatever the explanation may be, it seems significant that death occurred after both high and low doses of subcutaneous morphine, but only after high doses of subcutaneous codeine (cf. results). The possibility therefore exists that the lethal action of morphine is more complex in nature than that of codeine. Drug distribution studies could probably elucidate these problems and must await further experiments.

Summary

Nalorphine was a more potent antagonist to the analgesic action of codeine than to that of morphine. The pattern of antagonism differed, indicating an essentially different mechanism of morphine and codeine analgesia.

Death after morphine occurred in flaccid respiratory paralysis, whereas death after codeine was always associated with convulsions. Nalorphine was a potent antagonist to morphine lethality in the rat, but increased the lethal action of codeine. It was therefore concluded that the lethal actions of morphine and codeine in the rat are due to different mechanisms.

Simultaneous injections of morphine and codeine resulted in a potentiated action on analgesia and lethality in the rats. The possibility exists that morphine biotransformed from codeine and morphine injected into the animals may interact differently with codeine in the brain.

Intraperitoneal codeine was more toxic than subcutaneous codeine. Subcutaneous morphine exerted relatively greater lethal action at low than at high doses. For intraperitoneal morphine the reverse was true. The lethal action of morphine may be of a more complex nature than that of codeine.

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Tolerance to Morphine and Codeine Analgesia and Hepatic Enzymic Microsomal Drug Metabolism in the Rat

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In a previous study we found that chronic treatment with codeine can produce general reduction of enzymic drug metabolism in liver microsomes from rats (JÓHANNESSON, ROGERS, FOUTS & WOODS 1964) High doses of codeine were given, and the decreased drug metabolizing activities were therefore interpreted as being a non-specific result of tolerance rather than its cause. Nevertheless, the possibility existed that tolerance to morphine and codeine analgesia in the rat and the decreased drug metabolic activities in the liver microsomes were coincident phenomena and thus more or less causally related. In order to elucidate the relationship of these phenomena, tolerance to the analgesic action of morphine and codeine was produced in rats by high as well as by low doses. The rats were then killed, and their enzymic hepatic drug metabolism was compared with that of control rats (no drug administration) The metabolic pathways investigated represented some major pathways of drug metabolism, such as oxidation, reduction, dealkylation and glucuronide conjugation. Hepatic glycogen, a possible index of decreased microsomal enzymic activities (FOUTS 1963), was also determined

Methods

Animals. Male Holtzman rats (170–350 g) kept in constant environmental conditions at a room temperature varying from 22 to 26°C with free access to water and a commercial food preparation, were used. The experiments described were performed during June to October 1964

Injections. Morphine sulphate or codeine phosphate was dissolved in distilled water and administered by subcutaneous injection, given in the middle of the back in the midline, except when otherwise stated. Intraperitoneal injections were given in the middle of the abdomen t the midline. Intraperitoneal injections were given by t

culin syringes, calibrated to deliver 0.01 ml. The injected volumes amounted to 2.0 ml/kg, unless when the injections solution was 30 mg/ml of morphine sulphate in water (see below). All doses refer to the drugs as bases.

Analgesia was measured by the hot plate method, as modified by JÓHANNESSON & WOODS (1964). Analgesic effects were expressed qualitatively as the percentages of animals showing analgesia by the criteria used. During the performance of the experiments recorded here, it was found necessary to quantitate the responses of rats to the heat stimulus (see below) This was accomplished by calculating the mean reaction time for rats in a given group In experiments with the analgesics, rats were taken off the hot plate if they did not react in 30 seconds. Thirty seconds is therefore the highest score obtainable when quantitative results are given.

Experiments Four experiments were performed.

1 The first experiment, lasting 32 days, was performed to elucidate how soon tolerance to the analgesic action of morphine could be acquired by daily subcutaneous injections of the drug in low amounts. The injection solution was 30 mg/ml of morphine sulphate in water Injections were given in the gluteal region in the morning and in the evening, each dose amounting to half the daily dose.

Two experimental groups were used. Rats in the first group received a constant dose of approximately 13.5 mg/kg daily Rats in the second group received the same dose daily for 3 days then the daily dose was gradually increased to approximately 20.5 mg/kg during the next 7 days. The last daily dose of morphine was given to rats in both groups on the evening of the 10th day Rats in both groups were injected with a test dose of subcutaneous morphine (5 0 mg/kg) on the mornings of the 4th, 7th, 11th, 18th, 25th and 32nd days of the experiment. The analgesic effect was determined 30 minutes after the injections and expressed as the percentage of animals showing a positive analgesic reaction. After having been tested on the morning of the 4th and 7th day the rats were injected with additional amounts of morphine in order to make complete their scheduled morning dose of the drug. The experiment also included a group of rats given 13 5 mg/kg of morphine sc. daily for 8 days. On the 9th day the rats were killed for hepatic microsomal enzyme studies (see below).

The experiment included 2 control groups. Rats in the first group were injected with the test dose of morphine (5.0 mg/kg s. c.) once a week (on the 4th, 11th, 18th, 25th and 32nd days) and the analgesic effect was determined. Rats in a second control group were also injected weekly for 4 weeks. Morphine was, however only given at the first and last injections (4th and 32nd days) water alone (2.0 ml/kg) was injected on other days (11th 18th and 25th days). The rats, given either morphine or water were tested on the hot plate The second control group was included to evaluate the possible influence of time and repeated exposure to the hot plate procedure on the analgesic effect.

2. Unexpectedly rats in the first control group above, receiving a test dose of subcutaneous morphine once a week (5 injections), were found to develop tolerance to the analgesic effect of the drug A second experiment was then performed to substantiate this.

A number of rats were injected with the test dose of morphine weekly for four weeks, as had been the rats in the mentioned control group of experiment 1 However in order to be better able to estimate the onset of morphine tolerance, the analgesic effect was determined at intervals after the injections (cf JÓHANNESSON & WOODS 1964) and expressed as the mean reaction times in seconds (± the standard errors of the means) instead of as percentages of animals showing positive analgesic reponses. Two groups of control animals of the same age were run concurrently with the rats given

morphine once a week. Rats in one group received water only (2 ml/kg) once a week for 4 weeks (5 injections). Four weekly injections of water were similarily given to rats in a second control group the test dose of morphine being administered in the last injection. The rats were tested on the hot plate, whether they had received water or morphine, and their reaction times were read.

Tests were made to establish whether or not weekly injections of codeine might produce tolerance to the analgesic effect of the drug. Previous investigation had shown that 5.0 mg/kg of morphine s. c. is equal in analgesic potency to 60.0 mg/kg of codeine s. c. 30 minutes after the injections (JÓHANNESSON & WOODS 1964). Several rats were therefore given this dose of codeine once a week for 4 weeks (5 injections). The analgesic effect was determined at intervals after the injections, as in the rats given morphine.

Appropriate numbers of rats in each group, whether given analgesic or water were killed 16–20 hours after the last injections. The liver was removed for determination of glycogen and studies of hepatic microsomal drug metabolism.

3 The influence of the route of administration on tolerance development was investigated. Intraperitoneal morphine shows one-half the analgesic potency of subcutaneous morphine in tests performed 30 minutes after the injections (JÓHANNESSON & WOODS 1964). Several rats were therefore injected with 10 mg/kg of morphine i. p. once a week for 3 weeks (4 injections). The analgesic effect was determined and expressed as in experiment 2. Adequate groups of control rats of the same age were run concurrently with the rats given morphine. Appropriate numbers of rats in each group were killed for hepatic microsomal enzyme studies 16–20 hours after the last injections.

The possible effect of different time intervals between injections of morphine on the onset of tolerance to the analgesic action was also studied. Intraperitoneal morphine (10.0 mg/kg) was given to rats daily on 4 consecutive days, and the analgesic effects were determined and expressed in the same way as when the morphine was given once a week.

4 The fourth experiment was performed to evaluate hepatic enzymic drug metabolism and glycogen contents in rats treated with higher doses of morphine than had been used in the three previous experiments. The rats were given daily injections of morphine in increasing amounts for 4 weeks, as described by JÓHANNESSON & WOODS (1964). The last daily dose amounted to approximately 75 mg/kg. Adequate groups of control rats were run, and the animals were killed as described.

Preparation of tissue. Rats were killed by a blow on the head. The livers were immediately removed and homogenized on ice with a Potter homogenizer having a plastic pestle. The homogenates were prepared so that each gram of liver tissue was suspended in 2.0 ml of isotonic KCl (1.15 %) to give a total volume of approximately 3 ml. A supernatant fraction (9000 × g supernatant) containing microsomal and soluble enzymes, was prepared from the homogenates with a high speed angle centrifuge at 5°C.

In vitro determinations. One ml of the 9000 g supernatant was incubated in a Dubnoff metabolic shaker incubator at 37°C with oxygen, or nitrogen, as the gaseous phase, for determination of the enzymic activities. Final concentrations of the cofactors were triphosphopyridine nucleotide (NADP) 1.1×10^{-4}M glucose-6-phosphate 5×10^{-3}M, nicotinamide 2×10^{-2}M and $MgSO_4$ 5×10^{-3}. The last cofactor was omitted from benzpyrene pathway. The concentrations of all cofactors were supra-optimal. Final volumes were brought to 5.0 ml and adjusted to pH 7.4 with 0.1 M potassium phosphate buffer.

The pathways studied, the methods used in their assay and the substrate concentrations per 5 ml incubation mixture were

a) Reduction of the nitro-group of *p*-nitrobenzoic acid to yield *p*-aminobenzoic acid (FOUTS *et al.* 1957), 12.0 µmoles

b) ring sulphur oxidation of chlorpromazine (SALTZMAN & BRODIE 1956), 1.0 µmole

c) hydroxylation of benzpyrene to yield hydroxybenzpyrene (CONNEY *et al.* 1957), 0.6 µmoles

d) conjugation of "free" morphine to morphine glucuronide (glucuronide synthesis), 0.625 µmoles. The technique used was, with minor changes, that of TAKEMORI (1960). The disappearance of "free" morphine from the incubates was considered a measure of glucuronide formation. In order to check the specificity of the process, certain samples were hydrolysed by HCl. Essentially all of the morphine was recovered.

e) O-dealkylation of codeine to yield morphine (determined by the colour reaction of SNELL & SNELL (1937) the detailed analytical procedure is given below) 10.0 µmoles

f) N-dealkylation of aminopyrine to 4-aminoantipyrine (4-aminophenazone), 40.0 µmoles. The metabolite 4-aminoantipyrine was determined by the method of BRODIE & AXELROD (1950) as modified by GAUDETTE (cf. JÓHANNESSON, ROGERS, FOUTS & WOODS 1964)

Disappearance of substrate was measured in following the metabolism of chlorpromazine and benzpyrene and in glucuronide synthesis. Appearance of the metabolites listed was determined in the aminopyrine, *p*-nitrobenzoic acid and codeine metabolic pathways. Incubation times were 120 minutes except for synthesis of morphine glucuronide and benzpyrene hydroxylation (30 minutes) and for the nitro-group reduction (60 minutes)

In the procedure for determination of morphine, 4 ml of the incubation mixture were placed in 45 ml shake-tubes containing 0.2 ml of 2 M–Na_2CO_3 and 25 ml of chloroform (containing 10 % (v/v) of *iso*-amyl alcohol). The mixture was shaken for 10 minutes and centrifuged. Morphine was now found quantitatively in the chloroform phase. Morphine was transferred to the aqueous phase by shaking 20 ml of the chloroform phase with 7.0 ml of 0.1 N HCl for 10 minutes. The mixture was then centrifuged again, and 5.0 ml of aqueous phase were transferred to # 19 Coleman cuvettes (blank contained 5.0 ml of 0.1 N–HCl). Two ml of the silicomolybdate reagent (SNELL & SNELL 1937) and 2.0 ml of 50 % (w/v) NH_4OH were added to the cuvettes. The colour was allowed to develop for 15 minutes. The samples were read against the reagent blank on a Coleman Jr photometer at 650 mµ. Values were determined by comparison with a known amount of morphine, centrally located on a standard curve and taken through the analytical procedure during each determination. Standard curves were worked out with each new supply of reagents. The smallest measurable amounts are approximately 60 nmol of morphine sulphate.

Results are expressed as µmoles of the substrates metabolized, or the products formed, per g nitrogen per incubation period. Nitrogen was determined by a micro-Kjeldahl technique. The detailed analytical procedure is given below

One tenth ml of the 9000 × *g* supernatant microsomal fraction was transferred to boiling tubes containing 0.9 ml of distilled water and 1 selenized Henger granule for catalyzing the process (obtained from Henger Co., Philadelphia, Penn., U.S.A.). One ml of 50 % (w/v) H_2SO_4 was added, and the mixture was boiled under a hood for 30 minutes or until the solution was all white. The solution was allowed to cool and 1 drop of 30 % (v/v) hydrogen peroxide (superoxyl) was added. Boiling was repeated (for 10–15 minutes), and the solution was allowed to cool. The contents of the tube were then transferred to a 50 ml volumetric flask. The tube was washed several times with

distilled water and the volumetric flask was filled to the mark. Seven to 12 ml of the aqueous solution were placed in centrifuge tubes and centrifuged for sedimentation of the selenium. One ml of the clear solution was placed in each of several # 19 Coleman cuvettes, and five ml of a freshly prepared dilution of Nessler's stock solution (see below) were put into each cuvette. The contents were mixed and allowed to stand for 15 minutes for colour development. The optical density was read at 420 mμ on a Coleman Jr photometer (blank containing 1 ml water). The amount of nitrogen was determined by comparison with a NH_4Cl standard curve determined with each new batch of Nessler's solution (approximately every month). Any ammonia in the air will invalidate the determination. The tubes should therefore be kept stoppered as much as possible.

The Nessler's stock solution (labelled Bock and Benedict) was obtained from E. H Sargent & Co., Chicago Illinois, U.S.A. The active ingredient is a complex compound of potassium and mercury iodide, which reacts with ammonium, developing a yellow amber colour (cf ALLPORT & KEYSER 1957). The deluted reagent was made by adding 1 part of the stock solution to 5 parts of 1N–NaOH.

Glycogen determinations. A piece of liver (0.2–0.6 g) was excised and placed in 30 ⁄ (w/v) KOH solution as soon as possible after the animals had been killed. Glycogen was determined in the excised tissues by the phenol-suphuric acid method of MONTGOMERY (1957). Results are expressed as mg glycogen per g liver tissue.

Frequencies of occurrence of qualitative observations were compared by the χ^2 test (BURCH *et al.* 1950). Differences between two sample means were evaluated by the "t" test (SNEDECOR 1956). The level of significance was taken at $P \leq 0.05$.

Results

1 *Analgesimetry*

Experiment 1 The results are depicted in fig. 1 They show that the analgesic potency of a test dose of morphine (ED ⁄ 70–80) is rapidly lost when rats are given low amounts of morphine daily for 10 days (Group 1 & 2). From the 11th day until the end of the experiment, only about 10% (3–4 in number) of the animals in this group showed a positive analgesic response when tested. As far as could be determined, three of these animals were the same throughout the experiment.

The test dose only was given once a week for 4 weeks, and the analgesic effect was determined. The degree of analgesia measured after the last weekly injection was significantly lower than that after the first one (Group 3). A number of rats were injected twice, 28 days apart, with the test dose of morphine during this interval they were injected thrice with water and tested on the hot plate. The test dose exerted practically the same analgesic effect both times (Group 4)

At the beginning of the experiment, rats in all groups were of nearly the same weight, 170–185 g. Rats in Group 1 & 2 gained less in weight than rats in Group 3 This difference was statistically significant on the 7th and 11th day but not when the rats were weighed on the 18th day

The pathways studied, the methods used in their assay and the substrate concentrations per 5 ml incubation mixture were

a) Reduction of the nitro-group of *p*-nitrobenzoic acid to yield *p*-aminobenzoic acid (FOUTS *et al.* 1957), 12.0 μmoles

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In the procedure for determination of morphine 4 ml of the incubation mixture were placed in 45 ml shake-tubes containing 0.2 ml of 2 M–Na_2CO_3 and 25 ml of chloroform (containing 10 % (v/v) of *iso*-amyl alcohol). The mixture was shaken for 10 minutes and centrifuged. Morphine was now found quantitatively in the chloroform phase. Morphine was transferred to the aqueous phase by shaking 20 ml of the chloroform phase with 7.0 ml of 0.1 N–HCl for 10 minutes. The mixture was then centrifuged again, and 5.0 ml of aqueous phase were transferred to # 19 Coleman cuvettes (blank contained 5.0 ml of 0.1 N–HCl). Two ml of the silicomolybdate reagent (SNELL & SNELL 1937) and 2.0 ml of 50 % (w/v) NH_4OH were added to the cuvettes. The colour was allowed to develop for 15 minutes. The samples were read against the reagent blank on a Coleman Jr photometer at 650 mμ. Values were determined by comparison with a known amount of morphine, centrally located on a standard curve and taken through the analytical procedure during each determination. Standard curves were worked out with each new supply of reagents. The smallest measurable amounts are approximately 60 nmol of morphine sulphate.

Results are expressed as μmoles of the substrates metabolized, or the products formed, per g nitrogen per incubation period. Nitrogen was determined by a micro-Kjeldahl technique. The detailed analytical procedure is given below

One tenth ml of the 9000 × *g* supernatant microsomal fraction was transferred to boiling tubes containing 0.9 ml of distilled water and 1 selenized Henger granule for catalysing the process (obtained from Henger Co., Philadelphia, Penn., U.S.A.). One ml of 50 % (v/v) H_2SO_4 was added, and the mixture was boiled under a hood for 30 minutes or until the solution was all white. The solution was allowed to cool and 1 drop of 30 % (v/v) hydrogen peroxide (superoxyl) was added. Boiling was repeated (for 10–15 minutes), and the solution was allowed to cool. The contents of the tube were then transferred to a 50 ml volumetric flask. The tube was washed several times with

distilled water and the volumetric flask was filled to the mark. Seven to 12 ml of the aqueous solution were placed in centrifuge tubes and centrifuged for sedimentation of the selenium. One ml of the clear solution was placed in each of several # 19 Coleman cuvettes, and five ml of a freshly prepared dilution of Nessler's stock solution (see below) were put into each cuvette. The contents were mixed and allowed to stand for 15 minutes for colour development. The optical density was read at 420 μm on a Coleman Jr. photometer (blank containing 1 ml water) The amount of nitrogen was determined by comparison with a NH_4Cl standard curve determined with each new batch of Nessler's solution (approximately every month). Any ammonia in the air will invalidate the determination. The tubes should therefore be kept stoppered as much as possible.

The Nessler's stock solution (labelled Bock and Benedict) was obtained from E. H. Sargent & Co., Chicago, Illinois, U.S.A. The active ingredient is a complex compound of potassium and mercury iodide, which reacts with ammonium, developing a yellow amber colour (cf ALLPORT & KEYSER 1957) The deluted reagent was made by adding 1 part of the stock solution to 5 parts of 1N–NaOH.

Glycogen determinations A piece of liver (0.2–0.6 g) was excised and placed in 30 ⁄ (w/v) KOH solution as soon as possible after the animals had been killed. Glycogen was determined in the excised tissues by the phenol-sulphuric acid method of MONTGOMERY (1957) Results are expressed as mg glycogen per g liver tissue.

Frequencies of occurrence of qualitative observations were compared by the χ^2 test (BLISS et al 1950). Differences between two sample means were evaluated by the "t" test (SNEDECOR 1956). The level of significance was taken t $P \leq 0.05$)

Results

1 *Analgesimetry*

Experiment 1 The results are depicted in fig. 1 They show that the analgesic potency of a test dose of morphine (ED ⁄ 70–80) is rapidly lost when rats are given low amounts of morphine daily for 10 days (Group 1 & 2). From the 11th day until the end of the experiment, only about 10 ⁄ (3–4 in number) of the animals in this group showed a positive analgesic response when tested. As far as could be determined, three of these animals were the same throughout the experiment.

The test dose only was given once a week for 4 weeks, and the analgesic effect was determined. The degree of analgesia measured after the last weekly injection was significantly lower than that after the first one (Group 3) A number of rats were injected twice, 28 days apart, with the test dose of morphine during this interval they were injected thrice with water and tested on the hot plate The test dose exerted practically the same analgesic effect both times (Group 4).

At the beginning of the experiment, rats in all groups were of nearly the same weight, 170–185 g. Rats in Group 1 & 2 gained less in weight than rats in Group 3 This difference was statistically significant on the 7th and 11th day but not when the rats were weighed on the 18th (

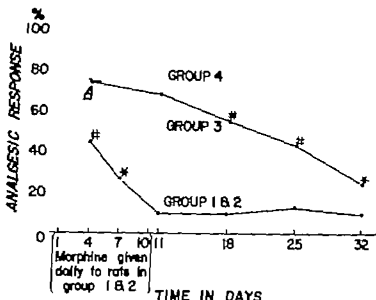


Fig. 1 Influence of repeated subcutaneous (s. c.) injections of morphine on the analgesic effect of rats in 4 groups, 16–18 rats in each group. Analgesia was determined at 30 minutes after the injections. *The abscissa* denotes time in days. The *ordinate* denotes percentage analgesic responses of rats in each group

Treatment Group 1 13.5 mg/kg of morphine administered daily during the first 10 days. *Group 2* Increasing daily amounts of morphine (13.5–20.5 mg/kg) during the first 10 days. Rats in groups 1 and 2 reacted in a practically identical manner and are therefore entered as one group (*Group 1 & 2*). A test dose of morphine (5 mg/kg s. c.) was given at intervals during and after the daily administration of morphine, and the analgesic effect was determined. *Group 3* the test dose was given at weekly intervals, and the analgesic effect was determined *Group 4* the test dose was given on the 4th day and at the end of the experiment, and the degree of analgesia was determined (for details, see experiment 1). A analgesic effect of the test dose when first given to rats in *Group 3* * denotes analgesic responses significantly (P < 0.05) lower than A and ≠ analgesic responses not significantly lower (P > 0.05) than A (X^2 test).

During the first week after discontinued daily administration of morphine to rats in Group 1 & 2, three rats in this group lost weight rapidly and died The mean weights of rats in Groups 3 and 4 never differed significantly When weighed on the last day of the experiment, mean weights of rats in the 3 groups did not differ significantly (means 317 317 and 337 g).

Two to three days after the experimental period, rats in Group 1 & 2 and Group 3 were mixed and then divided into 3 groups, 15 animals in each. Rats in one group were injected subcutaneously with 60.0 mg/kg of codeine a dose that has repeatedly given positive analgesic responses in 70–80% of animals tested (JÓHANNESSON & WOODS 1964 JÓHANNESSON 1964) and the analgesic effect was determined 30 minutes later Only 4 out of 15 rats showed positive analgesic effect. Rats in the second and third group were given 50% lethal doses of morphine or codeine by

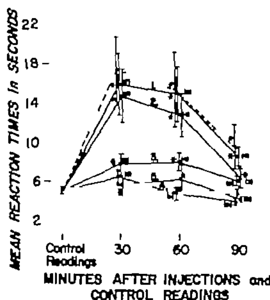


Fig 2. Influence of weekly subcutaneous injections of morphine on the analgesic effect. Three groups of rats, 9 animals in each, were used. Rats in one group were given 5.0 mg/kg of morphine once a week for 4 weeks (5 injections); rats in a control group received the water only (2 ml/kg). Rats in a second control group were given 4 weekly injections of water and then morphine (5 mg/kg) in the last injection (for details, see experiment 2).

Control reaction times were read immediately before the injections. Then reaction times were read at 30, 60 and 90 minutes after the injections (abscissa). The ordinate denotes mean reaction times of each group; vertical lines indicate standard errors of the means. Figures in brackets indicate numbers of animals showing analgesia by the criteria used.

Results of weekly morphine injections are indicated by thin, unbroken and numbered lines. A broken line refers to results of the 5th injection (morphine) to rats in the second control group. For sake of convenience, only one control reaction time is shown. It is the grand mean of all means. Reaction times of water controls are, for the same reason, entered (thick unbroken line) as means of pooled results at each period after all 5 injections. In statistical analyses ("t" test), means of results obtained from injections given on the same days are compared (means of 9 results each).

* mean value significantly higher than that of corresponding water control ($P < 0.05$).
\# mean value not significantly higher than that of corresponding water control ($P > 0.05$).

intraperitoneal injections (cf JÓHANNESSON 1964). No tolerance was observed to the lethal effect of codeine and only a slight tolerance, if any to morphine lethality

Experiments 2 and 3 Results of weekly injections of subcutaneous morphine are summarized in fig. 2. Although the occurrence of positive analgesic responses were not greatly different at 30 or 60 minutes after the first 3 injections, the mean reaction times were much lower after the third than after the two first injections. In contrast to the first and second injections, morphine given in the third injection thus hardly increased significantly the mean reaction times at 30 or 60 minutes (the 60 minutes value is just high enough to be significant t = 2.16) When morphine was

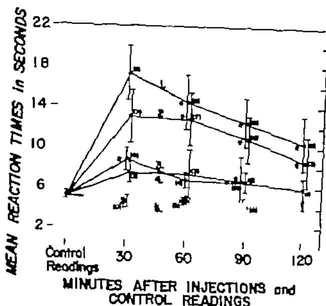


Fig. 3. Influence of weekly subcutaneous injections of codeine on the analgesic effect. Codeine was given in amounts of 60 mg/kg to 12 rats once a week for 4 weeks (5 injections). The analgesic effect was determined at 30, 60, 90 and 120 minutes after the injections (see fig. 2)

administered for the 4th and 5th time, most animals failed to show positive analgesic responses, and the reaction times approximated to those obtained after the injections of water. Only after the first injection of morphine did the mean reaction time at 90 minutes differ significantly from that of the water controls.

A number of rats were given 4 injections of water once a week and tested on the hot plate. Then at the 5th injection they received the test dose of morphine. Morphine exerted statistically the same analgesic potency on these rats as that observed after the first of 5 weekly injections (fig. 2).

Fig. 3 shows the results of experiments with weekly injections of codeine. The analgesic effect of subcutaneous codeine was more persistent than that of subcutaneous morphine. Even at 150 minutes after the first injection, reaction times of several of the codeine rats had not yet reached the pre-injection control level, whereas the reaction times of rats given morphine were, with one exception, back to the control level at 120 minutes (JÓHANNESSON & WOODS, unpublished results). The intensity of morphine and codeine analgesia was, however, practically the same when tested at 30 and 60 minutes. Repeated injections of subcutaneous codeine resulted in a rapid decrease in analgesic effect in a way similar to that found after subcutaneous morphine. The mean reaction times of the codeine rats were

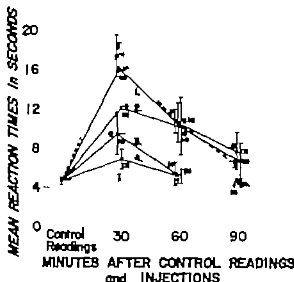


Fig 4. Influence of weekly intraperitoneal injections f morphine on the analgesic effect. Morphine was given in amounts of 10 mg/kg to 12 rats once week for 3 weeks (4 injections), and the analgesic effect was determined fter 30, 60 and 90 minutes. The control groups also contained 12 an mals. A broken line denotes the nalgesic effect of the morphine when given t rats in the second control group t the end of the experiment (see fig 2)

thus found not significantly higher after the third injection than those of the water controls. There were, on the other hand, still 6 out of 12 animals responding to the analgesic, by the criteria used, at 30 minutes after the third injection (fig. 3).

Results of experiments with weekly injections of intraperitoneal morphine are shown in fig. 4 Ten mg/kg morphine i. p exerted nearly the same analgesic effect as 5 mg/kg morphine s. c. or 60 mg/kg codeine s c. at 30 minutes after the first injection. At 60 minutes, morphine i. p appeared to show an indication, although not statistically significant one, of a lower effect than that observed after morphine or codeine s. c The analgesic effect declined rapidly upon repeated weekly administration It was already minimal at 60 minutes after the third injection (fig. 4) The persistence of morphine analgesia was the same whether the drug was given s. c. or i. p

Morphine (10 mg/kg) i. p was given daily on 4 consecutive days, and the analgesic effect was measured. It was rapidly lost and was found minimal even at 60 minutes after the second injection (fig. 5)

II. *Drug metabolism in the liver microsomes* The results are summarized in table 1 They show that chronic pretreatment with morphine in in-

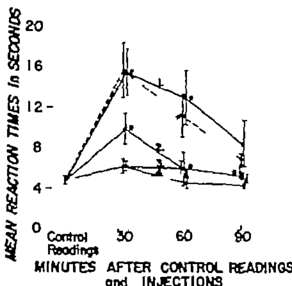


Fig. 5 Influence of daily intraperitoneal injections of morphine on the analgesic effect. Morphine in amounts of 10 mg/kg was given t 12 rats on 4 consecutive days, and the analgesic effect was determined (see figs. 2 and 4).

creasing amounts for 4 weeks (column 1) results in a significant decrease of drug metabolizing activities by hepatic enzymic microsomes in the rats. These results are similar to our previous results of chronic pretreatment with codeine for 4 weeks (JÓHANNESSON *et al* 1964) When a constant dose of morphine (ca. 13.5 mg/kg) was given daily for 8 days, 2 of 5 metabolic pathways studied were significantly inhibited (column 2). Columns 3–5 show the results of experiments in which equianalgesic doses of morphine s. c., codeine s. c. and morphine i. p were given to the rats once a week only for 3 or 4 weeks. No decrease in enzymic activities was found in these groups. Glucuronide synthesis was included in the study as the results of TAKEMORI & GLOWACKI (1962) suggested that morphine among phenolic compounds, might selectively inhibit glucuronyl transferase activity in rats.

Hepatic glycogen was decreased when the drug metabolizing activities were inhibited (columns 1,2).

Discussion and Conclusions

Seventy five per cent of rats given a test dose of subcutaneous morphine (5 0 mg/kg) showed analgesia by the criteria used in tests performed at 30 minutes after the first injection. The analgesic potency of the test dose decreased rapidly if the rats had been given daily injections of 10–20 mg/kg

Table 1

Hepatic microsomal drug metabolism and glycogen contents in rats treated with morphine or codeine. Microsomal enzymic activities are expressed as μmoles of substrates metabolized, or products formed, per g nitrogen per incubation period. Glycogen is expressed as mg per g wet liver. Each value represents the mean of determinations from 5–9 rats ± the standard deviation. Italicized figures denote the results of experiments with animals never given any drug.

Metabolic Pathways (Substrates – Metabolites)	Morphine Sc. daily for 4 weeks (cf Experim. 4)	Morphine Sc. daily for 8 days (cf Experim. 1)	Morphine Sc. weekly for 4 weeks (cf Experim. 2)	Codeine Sc. weekly for 4 weeks (cf Experim. 2)	Morphine I.p. weekly for 3 weeks (cf Experim. 3)
Aminopyrine → 4-Aminoantipyrine	12 ± 2 *20 ± 4*	12 ± 2 *19 ± 4*	16 ± 3 *23 ± 2*	16 ± 3 *18 ± 3*	16 ± 2 *22 ± 4*
p-Nitrobenzoic Acid → p-Aminobenzoic Acid	27 ± 18* *64 ± 10*	29 ± 4 *35 ± 10*	48 ± 15 *53 ± 11*	43 ± 11 *48 ± 9*	31 ± 5 *30 ± 10*
Codeine → Morphine	31 ± 10* *81 ± 8*	89 ± 12 *91 ± 10*	108 ± 14 *105 ± 11*	83 ± 6 *88 ± 7*	77 ± 10 *93 ± 12*
Chlorpromazine → Sulfoxychlorpromazine	6 ± 2* *61 ± 12*	20 ± 7* *41 ± 8*	60 ± 10 *52 ± 8*	57 ± 9 *63 ± 15*	51 ± 8 *56 ± 7*
Benzpyrene → Hydroxybenzpyrene	4 ± 2 *14 ± 8*	4 ± 2 *13 ± 3*	15 ± 4 *13 ± 2*	13 ± 4 *12 ± 2*	–
Morphine → Morphine Glucuronide				17 ± 3 *21 ± 4*	16 ± 4 *18 ± 3*
Hepatic Glycogen	44 ± 8 *62 ± 7*	40 ± 5 *69 ± 8*	57 ± 9 *55 ± 5*	56 ± 9 *61 ± 10*	59 ± 8 *57 ± 9*

* Values significantly lower than the respective control values.
Sc. Injected subcutaneously. I.p. Injected intraperitoneally.

of morphine. Even when no morphine except the test dose itself was administered to the rats, with a week's interval between injections, the frequency of positive analgesic responses was found significantly lower after the fifth than after the first injection. Time and the hot plate procedure for testing of analgesia were ruled out as factors causally related to the decreased analgesic effect of morphine (fig. 1). The results therefore

show that tolerance to morphine analgesia in the rats could be elicited by *very few and low doses* of the drug.

Although the percentage analgesic responses after subcutaneous morphine were appreciably lower after the third and fourth weekly injections, the number of animals exhibiting analgesia at 30 minutes were not significantly lower than after the first injection (fig. 1) In a second experiment, in which the morphine was also administered once a week, morphine analgesia was expressed as the mean reaction time of a group of rats on the hot plate. The results showed that only after the two first injections was the mean reaction time of rats given morphine significantly higher at 30 minutes than that of the water controls (fig. 2). Similar results were obtained in experiments with codeine s. c. and morphine i. p. in doses equipotent with 5 0 mg/kg of morphine s. c. at 30 minutes after the injections (figs. 3 and 4) The first sign of developing tolerance to morphine and codeine analgesia is thus *shortening of the effect*

Morphine analgesia declines rapidly between 60 and 90 minutes (figs. 2 and 4) but the analgesic effect of codeine s c. is still significantly above the control level at 120 minutes after the first injection (fig. 3). Whether the analgesic effect lasted for a longer or a shorter time after the first injection, it was rapidly lost on repeated weekly administration of the drugs. The analgesic responses showing the lowest statistical significance were lost first. The greatest decline occurred in these experiments *after the second injection* (figs. 2, 3 and 4). In experiments in which morphine i. p. (10 0 mg/kg) was given daily on 4 consecutive days, the analgesic effect decreased even more rapidly than when the same dose was administered in 4 weekly injections. The greatest decline was observed after the first daily injection (fig. 5) The possibility therefore exists that the intervals between the injections of a given dose of morphine may influence the degree of tolerance developed to morphine analgesia.

Since the beginning of scientific research on morphine tolerance about 80 years ago rats have been widely used test objects. Few investigators have, however performed experiments to elucidate the dosages or the *periods of administration necessary to develop tolerance* to the various actions of morphine in rats. Without giving any further details, JOËL & ETTINGER (1926) stated that rats showed signs of tolerance to the narcotic effect of morphine after only 3–4 daily injections of the drug (about 50 mg/kg s. c. a day) Their results indicated that tolerance to morphine, once aquired, persisted. SIMON & EDDY (1935) found that the effect of morphine on the performance of rats running an elevated maze decreased after a few weekly injections of the drug (about 10 mg/kg s. c. or i. p. a dose) However analyses of the results indicated that the decreased susceptibility to morphine was causally connected with repeated maze-

running and not due to development of tolerance to morphine itself While the experiments recorded here were in progress, COCHIN & KORNETSKY (1964) *published their well documented results of experiments* on morphine tolerance in rats. They found that a single injection of intraperitoneal morphine sulphate (20 0 mg/kg) might result in a certain degree of tolerance to morphine analgesia when the rats were tested 3 months later The tolerance thus induced was extremely persistent and lasted for at least a year COCHIN & KORNETSKY found, on the other hand that much higher doses were needed to develop tolerance to the effect of morphine on the performance of rats in a swimming test. It is here of interest that, whereas tolerance to the lethal and analgesic effects of morphine was easily demonstrated in rats chronically treated with increasing daily doses of morphine (cf JÓHANNESSON & LONG 1964 and JÓHANNESSON & WOODS 1964), only tolerance to the analgesic effect was observed in the experiments recorded here (cf experiment 1) All available information therefore strongly indicates that tolerance to morphine analgesia in the rat is not only easily acquired, but is also of great persistence. The information further indicates that tolerance to other perhaps *all* other actions of morphine is not as easily acquired as that to the analgesic action. It is accordingly reasonable that different dosage schedules should be adopted in studying development of tolerance to the various actions of morphine in rats.

It has previously been shown that chronic pretreatment with high doses of codeine *may significantly decrease the drug metabolizing activities* of the hepatic microsomal enzymes in rats (JÓHANNESSON, ROGERS, FOUTS & WOODS 1964) The results of our investigation indicate that only chronic pretreatment with morphine or codeine will in rats significantly depress the drug metabolizing capacity of the liver microsomes (table 1) Four or five weekly injections of morphine or codeine in low amounts, which result in tolerance to the analgesic effect, were thus not found to affect significantly the metabolic pathways studied here. The decreased drug metabolizing activities of the liver microsomes were further paralleled by reduced concentrations of liver glycogen. Although hepatic glycogen may be a valuable index of depressed drug metabolizing enzyme activities in the liver microsomes, *its concentration is reduced in a number of* different physiological and pathological conditions (FOUTS 1963) In conclusion it can therefore be said that tolerance to morphine or codeine analgesia in the rat is *not likely* to be causally connected with altered drug metabolism in the liver microsomes. This view is supported by the work of CLOUET & RATNER (1964) in so far as they could not relate changes in the microsomal N-demethylase activity to development of morphine tolerance in rats. It is, on the other hand, still a question whether or not

tolerance to the analgesic action is causally connected with one or more of the enzymic activities in the rat s body These activities should therefore be investigated only when few and low doses of morphine or codeine have been administered to the animals, e g after the second or third weekly injection of a given test dose (see above). KEESER (1933) has already postulated that abstinence symptoms might be due to derangement of certain enzymic activities in the organism. If this is true, which is conjectural at the present time, the kind of abstinence symptoms demonstrated after the lowest possible doses of morphine could prove to be keystones to a better understanding of the more complex effects of morphine.

Summary

Tolerance to morphine and codeine analgesia in the rat can be produced by very few and low doses of the drugs. Cross-tolerance was also observed. Higher doses are needed to produce tolerance to morphine lethality

Tolerance to morphine and codeine analgesia and decreased drug metabolizing activities in the liver microsomes are not coincidental phenomena. The drug metabolizing activities are decreased only after more or less prolonged administration of morphine or codeine The decreased enzymic activities are paralleled by reduced concentrations of hepatic glycogen.

The first sign of developing tolerance to morphine or codeine analgesia in the rat is shortening of the effect. Tolerance to morphine analgesia was developed more rapidly when a test dose of the drug was given daily than when given once a week.

Different dosage schedules should be adopted in producing tolerance to the various actions of morphine and codeine in the rat.

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Failure of Reserpine to Deplete Noradrenaline Neurons of α Methylnoradrenaline Formed from α Methyl DOPA

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The marked and sustained depletion of tissue noradrenaline by α methyl DOPA (DOPA = 3 4-dihydroxyphenylalanine) is due to displacement of the amine by its analogue α-methylnoradrenaline, which is formed by decarboxylation of the amino acid and subsequent β-hydroxylation (CARLSSON & LINDQVIST 1962) Later work (CARLSSON & LINDQVIST unpublished results MUSCHOLL & LINDMAN 1964 SCHÜMANN & GROBECKER 1964) showed that the α-methylnoradrenaline formed in brain was caused to disappear by reserpine much less readily than was noradrenaline. This may be partly because of resistence to monoamine oxidase. The metaraminol formed from α methyl-*m*-tyrosine behaves similarly In fact, it is affected by reserpine even less than is α-methylnoradrenaline, which may be due to resistence also to catechol-O-methyl transferase. However an additional factor such as a high affinity for the binding sites of the storage granules, may partly explain the difference.

The work reported here was undertaken to see whether these phenomena could be utilized for a histochemical differentiation between dopamine and noradrenaline. Although other means exist for doing this (CARLSSON, FALCK & HILLARP 1962), there is a clear need for additional criteria.

Method

Sprague-Dawley rats (150–250 g) were injected with α-methyl DOPA in a single dose of *400 mg/kg intraperitoneally or in two such doses at a* 3 hours interval. Twenty-four (in a few experiments 48) hours after the first dose, reserpine was given intraperitoneally at a dose of 3 5 or 10 mg/kg. The animals were killed after another

Table 1

Animal treatments

α-methyl DOPA 400 mg/kg No. of doses	*Reserpine* mg/kg	*Killed* at hours fter reserpine treatment (no. of animals in brackets)
1	3	24 (4)
1	5	3 (2) 7 (2), 4 (8)
2	5	½ (2), 3 (4) 7 (2)
1	10	24 (4)
2	10	3 (3), 11 (2), 24 (3)
2	10 *)	3 (3), 24 (3)

*) Reserpine given 48 hr after first dose of α methyl DOPA, instead f 24 hr as I the other experiments.

½ 3, 7 or 24 hours (see table 1). Control animals treated with reserpine alone were always run in parallel.

Histochemical detection of tissue monoamines was performed as described in detail by DAHLSTRÖM & FUXE (1964). Extensive parts of the forebrain lower brain stem and spinal cord were examined.

Results

In animals pretreated with α methyl DOPA, in contrast to normal animals, reserpine (3 5 or 10 mg/kg) left the greenish fluorescence typical of *catecholamines* essentially unaffected in the *nerve terminals* (with the exceptions indicated below) In the *nerve-cell bodies* reserpine caused a reduction in fluorescence within 3 hours in some experiments (particularly in groups A 1 and A 8–A 10, see DAHLSTRÖM & FUXE 1964) but even then the effect of reserpine was weaker than normal in most areas. In the controls the green-fluorescent cell bodies lost their fluorescence entirely within 3 hours. Seven and 24 hours after reserpine treatment the cell bodies of the α-methyl DOPA animals (and the control animals) had regained their normal fluorescence.

In certain areas containing green-fluorescent nerve terminals the action of reserpine was apparently unaffected by pretreatment with α methyl DOPA. These areas were the *caudate nucleus-putamen*, the *median eminence* the *nucleus accumbens* and the *tuberculum olfactorium* In the two areas first-mentioned, the fluorescence disappeared almost entirely and in the other two it was markedly reduced. The effect was clear-cut already after ½ hr and fully developed after 3 hr., and it persisted after 24 hr In the course of the experiments one more area was discovered where reser-

initially after administering their respective precursors, but they disappear fairly rapidly without giving rise to the corresponding β-hydroxylated metabolites, α-methylnoradrenaline and metaraminol, respectively. This will explain why the green-fluorescent nerve terminals of the caudate nucleus-putamen showed a normal sensitivity to reserpine, when given 24 hr after treatment with α-methyl DOPA. The conclusion seems reasonable that the other areas with normal reserpine sensitivity i. e. the median eminence, the nucleus accumbens, the tuberculum olfactorium and the dorsal part of the nucleus interstitialis striae terminalis, also contain dopamine-storing nerve-terminals. These areas, incidentically have one other property in common with the caudate nucleus-putamen their nerve terminals are densely packed and extremely delicate, yielding an impression of diffuse fluorescence of the whole area. In fact, recent results (ANDÉN, unpublished) show that dopamine is the dominating catecholamine in the tuberculum olfactorium.

It should be noted that the picture obtained after α methyl DOPA plus reserpine is precisely the inverse of that obtained after α methyl-m-tyrosine.

Also the areas normally storing dopamine and noradrenaline behaved differently in their cell bodies after α-methyl DOPA treatment the dopamine cells of the substantia nigra were more sensitive to reserpine than the noradrenaline cells. This was particularly apparent in the experiments with a 48-hr interval between administering the two drugs.

The low diffusion tendency of α methylnoradrenaline observed in this investigation suggests that this analogue is more tightly bound to the storage sites than noradrenaline. This, in conjunction with resistence towards monoamine oxidase, may explain why α methylnoradrenaline causes such a pronounced and sustained depletion of noradrenaline. Whether this phenomenon may also contribute to explain the hypotensive and other pharmacological actions of α methyl DOPA remains to be seen. It should be recalled that ANDÉN (1964) found metaraminol to be not so easily extractable from tissues as noradrenaline.

In an earlier publication (CARLSSON & LINDQVIST 1962) we have suggested that the α-methylnoradrenaline formed from α-methyl DOPA and stored in noradrenaline neurons might take over some of the functions of the physiological transmitter In support of this MUSCHOLL & MAÎTRE (1963) have observed that stimulation of the sympathetic nerves of rats pretreated with α-methyl DOPA may result in release of α-methylnoradrenaline. The observation made in this and earlier investigations (for references, see CARLSSON 1964) that animals pretreated with α-methyl DOPA show a reduced sensitivity to the pharmacological actions of reserpine, argues to the same effect It also suggests that the reserpine

syndrome is partly a consequence of an interference with the transmitter function of noradrenaline.

Summary

Histochemical observations on rats treated with α methyl DOPA supported the view that the marked and sustained depletion of noradrenaline caused by this drug is due to displacement by α-methylnoradrenaline. This analogue shows the same histochemical reaction as noradrenaline. The α-methylnoradrenaline replacing its physiological analogue in nerve terminals of the brain was found to be resistent to reserpine, as indicated by persistence of fluorescence typical of catecholamines. The fluorescence of dopamine-containing nerve terminals, on the other hand, disappeared at a normal rate after reserpine treatment of animals pretreated with α-methyl DOPA. This provides an additional tool in the histochemical differentiation between noradrenaline and dopamine.

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Site of Action of Reserpine

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It has been known for almost 10 years that reserpine depletes the monoamine stores of the tissues, but opinion still differs widely as to the site and mode of action of this drug. With the help of a highly sensitive fluorescence method for the direct microscopical demonstration of catecholamines (CA) and 5-hydroxytryptamine (5-HT) the mechanisms for the uptake, storage, release and inactivation of these amines can now be studied in entirely new ways, as recently shown in this laboratory (DAHLSTRÖM & FUXE 1964a HAMBERGER *et al* 1964 FUXE & SEDVALL 1964 HILLARP & MALMFORS 1964 MALMFORS 1964 MALMFORS & SACHS 1965)

Studies on the peripheral adrenergic neuron (see MALMFORS 1965 NORBERG & HAMBERGER 1964) show that not only the terminals but the entire neuron has the basic property of taking up noradrenaline (NA) and related amines by means of an efficient uptake-concentration mechanism different from that of the amine storage granules (cf EULER 1958 EULER & LISHAJKO 1963 & 1964 CARLSSON, HILLARP & WALDECK 1963) and [illegible] localized in the cell membrane These studies further provided the [illegible] direct evidence that this mechanism is insensitive to reserpine but [illegible] inhibited by several other drugs (*e g* desmethyl-imipramine, [illegible] chlorpromazine and cocaine) (MALMFORS 1965). These facts [illegible] support the view (CARLSSON, HILLARP & WALDECK 1963) that [illegible] acts specifically and primarily by blocking the Mg++ ATP [illegible] uptake-storage mechanism in the amine granules. Further [illegible] this view has now been obtained in a different way by [illegible] depletion and recovery of CA and the formation of [illegible] central monoaminergic neurons (see DAHLSTRÖM & [illegible] 1965) after administration of reserpine or other drugs [illegible] or long-lasting depletion of the neuronal CA.

Table 1

Drug	Animals, Doses and Time before killing the animals
1 Reserpi	a) Rat ½ 1 2, 4 6, 8 12, 24, 48, 72 and 96 hours after a dose of 10 mg/kg. b) Rat: 4 hours after a dose of 0.05, 0.1 0.2, 0.5, 1 5 or 10 mg/kg. c) Mouse ½ to 96 hours after a dose of 25 mg/kg.
2. Tetrabenazine	a) Rat 2, 4 6, 7 8 12 and 20 hours after a dose of 50 or 100 mg/kg.
3 m-Tyrosine	a) Rat and mouse 2, 4 and 6 hours after the last of 3 doses of 400 mg/kg administered at 2 hourly intervals.
4 α-Methyl-m-tyrosine	a) Rat nd mouse 7 10, 13 16 and 22 hours after the last of 2 doses of 400 mg/kg administered with a 2 hours interval. b) Rat 6 nd 12 hours after a dose of 800 mg/kg.
5 Nialamide	) Rat 4–6 hours after a dose of 50, 100, 250 or 500 mg/kg. b) Rat 1 2, 3, 4 18 and 24 hours after a dose of 100 or 500 mg/kg. Half of the animals were pretreated with reserpine (5 mg/kg) 20 hours before administration of nialamide. c) Rat 1 2, 3 and 4 hours after a dose of 50 mg/kg. Half of the animals were pretreated with reserpine (10 mg/kg) 2 or 24 hours before administration of nialamide. d) Mouse 2, 4 and 6 hours after a dose of 25 or 50 mg/kg. Half of the animals were pretreated with reserpine (25 mg/kg) 2 or 20 hours before dministration of nialam de.

Materials and Methods

The CA and 5-HT nerve cells and terminals in the brain stem (and also many in the spinal cord) were examined from some 300 male Sprague-Dawley rats weighing 200–250 g (about 2 months old). Amine depletion and recovery were studied after intraperitoneal administration of various drugs (see table 1). At least 3 (usually 6) animals were used at each time or for each dose. They were killed by beheading under light ether anaesthesia in all experiments along with untreated control animals. White mice (20–30 g) were also used in large numbers.

The brain was taken out and dissected immediately after killing. The specimens were rapidly frozen in liquid propane, cooled by liquid nitrogen, freeze-dried, treated with formaldehyde gas, embedded in paraffin and sectioned, etc. as described in detail in a previous paper (DAHLSTRÖM & FUXE 1964a).

The fluorescence reaction (FALCK *et al.* 1962) is based on the principle that under certain conditions, primary CA (dopamine and NA) and 5-HT can be readily converted into 6,7-dihydroxy-3 4-dihydroisoquinolines and 6-hydroxy-3 4-dihydro-β-carbolines

(CORRODI & HILLARP 1963 & 1964), respectively. Owing to the intense fluorescence of these products, the presence of CA and 5-HT is visualized by what, in the fluorescence microscope used, are green (to yellow-green) and yellow fluorescences, respectively. The reaction is so sensitive that changes in the content and distribution of the amines can be readily studied, at least in the CA neurons.

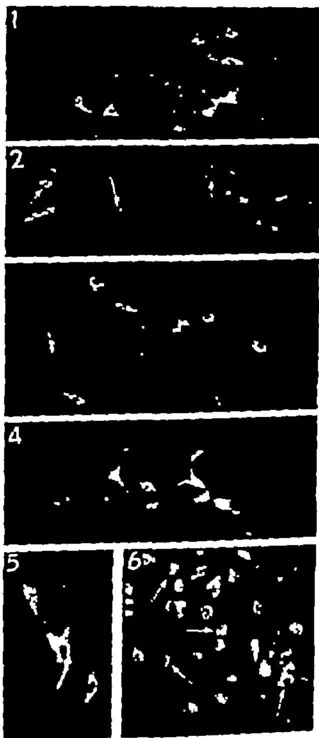
Results

The intraneuronal distribution of the amines in the central CA and 5-HT neurons has been described in previous papers (DAHLSTRÖM & FUXE 1964a, FUXE 1965). The cell bodies have a low amine content (probably in the range of 10–100 µg/g wet weight; see also NORBERG & HAMBERGER 1964) and exhibit a weak to strong fluorescence in the entire perinuclear cytoplasm. The zone around the nucleus sometimes shows higher accumulations. The presynaptic structures (the varicosities) of the terminals store the amines at extremely high concentrations (probably of the order of 10,000 µg/g). The non-terminal axons seem to have somewhat higher concentrations than the cell bodies, but exhibit at the best only a weak fluorescence because of their thinness.

The changes observed in the contents and intraneuronal distributions of the amines after administration of reserpine and other drugs (for doses and times, see table 1) are briefly described below. No obvious differences were found between rat and mouse. In the depletion-recovery experiments, there are given only the observations made on the CA neurons, since the cell bodies and terminals are much easier to examine in these than in the 5-HT neurons. The specificity of the fluorescence reaction has been discussed in detail in a previous paper (DAHLSTRÖM & FUXE 1964a).

Reserpine. The CA contents of the nerve cell bodies were markedly decreased as early as 30 minutes after administration of a large dose. Complete depletion was obtained after 2 or 4 hours. The various groups of cells showed marked differences in recovery. A more or less marked recovery was, however, observed, especially in the A1 and A2 cells after 6–12 hours, and by 24 hours numerous cells had recovered almost completely or showed a normal appearance. The CA in the nerve terminals disappeared at about the same rate, but the earliest sign of recovery – a weak fluorescence in the varicosities of some terminals – was observed first after 72 hours. A slow increase then occurred over several days.

A dose of 1 mg/kg (rat) produced a distinct fall in CA levels in the mesencephalic nerve cells, but the cells in the medulla oblongata were for the most part unaffected. The reduction affected all parts of the cell bodies and their processes. A dose of 5 mg/kg usually led to total depletion. The terminals showed about 5–10 times higher sensitivity to


1
2
4
5
6

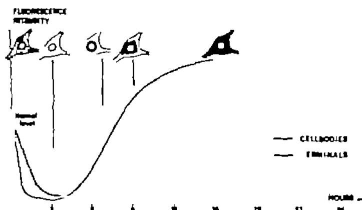


Fig 7 Schematic illustration of the depletion and recovery of the catecholamines in the cells bodies and terminals of nerve cells belonging to group A1 after administration of a large dose (10 mg/kg) of reserpine. The recovery begins rapidly with amine accumulation in a zone around the nucleus. In the later stages, amines begin to accumulate in the entire cell body and are finally found in the processes also ▦, Strong fluorescence ⊞, □ Medium fluorescence ▪ Weak fluorescence □, No fluorescence.

After total depletion, CA began as a general rule to reappear first in a zone around the cell nucleus. This was best seen in cells showing a rapid recovery (*e g* the cells of group A1 see DAHLSTRÖM & FUXE 1964a) The CA in this zone usually reached normal levels (or in group A1 even levels above normal, with large perinuclear accumulations) before any more marked recovery could be observed in the outer part of the cell body Last to regain their CA content were the cell processes (fig. 1–6) The time course of depletion and recovery is schematically represented in fig. 7

Tetrabenazine A large dose of this drug produced complete depletion of CA from both cell bodies and terminals after 2–4 hours Recovery was

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Fig. 1 Normal rat. Group A1 NA nerve cells together with CA terminals. One of the cells has higher concentration of amines around the nucleus (→) 165

Fig 2. Reserpine treatment, 5 hours. Group A1 Accumulation of newly formed CA is observed mainly in the perinuclear zone of the cells (→). 165

Fig. 3. Reserpine treatment, 8 hours. Group A1 Marked accumulation of CA is observed. The entire cell body is almost filled. 165

Fig. 4 Reserpine treatment, 24 hours Group A1 The newly formed CA fill the entire cell body and have reached supernormal levels (→). 165 ×

Fig. 5. Reserpine treatment, 12 hours. Group A1 The CA rich zone around the nucleus is large and has almost reached the processes of the cells. 460 ×

Fig 6. Reserpine treatment, 6 hours. Group A10. Newly formed CA have accumulated, especially in a zone around the nucleus (→). Recovery at this stage has just begun, but appears in the picture to be fairly marked owing to a long exposure. 250 ×

rapid and occurred simultaneously in both nerve cells and terminals. All the CA cells exhibited a weak specific fluorescence in the entire cell body after 6–7 hours. The cells and terminals showed somewhat reduced CA levels after 8–12 hours but were otherwise of normal appearance. Recovery was complete after 20 hours.

m-Tyrosine. Both the cell bodies and terminals of the CA neurons were almost completely depleted 2 hours after administration of the last dose. Recovery was rapid and occurred simultaneously in both cells and terminals. As early as 4 hours after the last dose, all parts of the cell bodies and processes showed a marked recovery and 2 hours later their appearance was normal. As after the administration of α-methyl-m-tyrosine (see below), the cell bodies did not usually show total depletion, but exhibited a weak to extremely weak diffuse fluorescence, which then gradually increased.

α-Methyl-m-tyrosine (α-MMT). Both the cell bodies and terminals of the CA neurons were almost completely depleted 7 hours after administration of the last dose. The cells in the large mesencephalic groups recovered most rapidly (within 13–16 hours) and those of most other groups recovered within 22 hours. As was found, although much more pronouncedly in the reserpine experiments, recovery could be seen in several instances to begin with an accumulation of CA in a zone around the cell nucleus. Amines then appeared in the outer parts of the cell bodies too and finally in the processes. The dopamine (DA) terminals also showed a rapid recovery but the NA terminals were still depleted 22 hours after the last dose (for details, see FUXE 1965). Of special significance is the finding that the NA cells of group A1 recovered within 22 hours, in contrast to their terminals in the spinal cord. Six hours after administration of a large dose (800 mg/kg), the cell bodies and terminals of the CA neurons were almost completely depleted. However, several of the cells, especially in groups A1, A2 and A4, showed a distinct accumulation of CA in the perinuclear zone. The cells in these groups recovered somewhat more slowly than the mesencephalic cells, which showed almost normal CA levels in the entire perikaryon after only 12 hours. At this time a marked accumulation of CA was found around the nucleus in numerous A4 cells. The cells of groups A5–7 were still depleted, but could often be seen to have a just perceptible perinuclear accumulation of CA.

Nialamide. Potent monoamine oxidase (MAO) inhibitors of entirely different chemical structures produce marked increases in amine levels in the cell bodies, axons and terminals of the 5-HT neurons in rat and mouse (DAHLSTRÖM & FUXE 1964a, BARTONIČEK, DAHLSTRÖM & FUXE 1964).

After administration of a large dose of nialamide (experiment 5b in

table 1), increased 5-HT levels were observed first in the cell bodies and especially in a zone around the nucleus (1 hour) and then also in the axons and terminals (2 hours). After 3–24 hours, levels were much higher in all parts of the neuron. Doses of 25 and 50 mg/kg were sufficient to give a pronounced increase after 4 hours.

In three experiments (5 b–d in table 1), the effects of low or high doses of nialamide were examined in both normal and reserpinised animals. A large dose of reserpine was administered either 2 or 20–24 hours before the nialamide. No obvious differences between normal and reserpinised animals were found in increase in 5-HT levels of the cell bodies or non terminal axons. The terminals were difficult to compare, as they normally have much higher 5-HT concentrations, but the terminals in the reserpinised animals showed a rapid accumulation of 5-HT and high to extremely high levels (comparable to those normally existing) were obtained after 3–4 hours, depending on the dose of nialamide.

Discussion

From observations made on amine uptake *in vitro* by blood platelets and tissue slices it has been concluded that the amines are incorporated and maintained in the tissue stores by an active transport mechanism or "pump" which is assumed to be localized in the cell membrane (for references, see SHORE 1962, BRODIE & BEAVEN 1963). It has been further suggested that the primary action of reserpine on amine stores is to block this pump irreversibly. The blockade leads to a depletion of the stores as the amines leave by passive diffusion.

There is convincing evidence that adrenergic nerves can take up and store exogenous NA and adrenaline (for references, see ANDÉN 1964a, STJÄRNE 1964). The fact that little administered NA is retained in the tissue of reserpinised animals has been taken as further evidence that reserpine blocks amine uptake (see MUSCHOLL 1960, SHORE 1962, STJÄRNE 1964).

There is now also strong evidence, however, that reserpine acts specifically and primarily by blocking the storage mechanism of the amine granules and not by blocking a pump in the cell membrane (cf. CARLSSON 1964a). Since opinions in this field still differ widely, a discussion of the more relevant information would seem in place. A summary account will also be given of recent observations made by more direct methods in our laboratory.

It should first be emphasized that the assumption of 5-HT existing free in the platelets, which is in fact the basis for the conclusion that 5-H

maintained by a reserpine-sensitive transport mechanism, has been shown to be wrong (BUCKINGHAM & MAYNERT 1964).

Studies on the intracellular localization of DA, NA and 5-HT formed in the adrenal medulla from administered 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan furnished the first direct evidence that reserpine *in vivo* inhibits the storage mechanism of the amine granules (BERTLER, HILLARP & ROSENGREN 1960 & 1961, CARLSSON, JONASSON & ROSENGREN 1963). These granules are able to take up and concentrate monoamines *in vitro* by a specific Mg^{++} ATP-dependent storage mechanism, which is blocked by extremely low concentrations of reserpine (CARLSSON, HILLARP & WALDECK 1962 & 1963, KIRSHNER 1962). This drug is the strongest inhibitor known so far and the high specificity of reserpine in acting at some vital point in the storage mechanism is demonstrated by the finding that isoreserpine is about 100 times weaker (CARLSSON, HILLARP & WALDECK 1963). From these and other results it was concluded that two different mechanisms of amine uptake operate in monoamine storage cells at the same time, namely a reserpine-insensitive transport mechanism probably located in the cell membrane and a mechanism highly sensitive to reserpine and located in the storage granules. The latter mechanism is blocked in granules isolated from the medulla of reserpinised animals (KIRSHNER, RORIE & KAMIN 1963, LUNDBORG 1963), and reserpine competes with CA for the same groups in the uptake mechanism (JONASSON, ROSENGREN & WALDECK 1964). The significance of these observations is emphasized by the fact that the transmitter of the adrenergic nerves is stored in granules with similar properties (EULER & HILLARP 1956, EULER 1958, EULER & LISHAJKO 1961, SCHÜMANN 1958). The nerve granules also show a Mg ATP-dependent uptake of NA *in vitro* which is blocked by reserpine (EULER & LISHAJKO 1963 & 1964, STJÄRNE 1964). As expected from the fact that the medullary cell and the adrenergic neuron are closely related cells, the effects of reserpine on the adrenal medulla and adrenergic nerves are closely similar in essential points, which is further emphasized by recent observations (CARLSSON, JONASSON & ROSENGREN 1963, ANDÉN, MAGNUSSON & WALDECK 1964). There is thus strong support for the conclusion that reserpine blocks the specific storage mechanism in the nerve granules also. Further evidence has been obtained in experiments indicating that the adrenergic transmitter is to a large extent destroyed by MAO within the nerves after the administration of reserpine (CARLSSON *et al.* 1957, KOPIN & GORDON 1962, KOPIN, HERTTING & GORDON 1962). Balance studies on the removal, net uptake and metabolism of NA infused into the isolated perfused heart have shown that reserpine does not prevent the removal of the amine by the heart, although no net uptake occurs, owing to enzymatic inactivation (KOPIN,

HERTTING & GORDON 1962 LINDMAR & MUSCHOLL 1964) These and other findings give considerable weight to the conclusions of LINDMAR & MUSCHOLL that there are two mechanisms concentrating NA, namely a transport across the cell membrane and an uptake into storage sites, and that reserpine blocks the latter

The uptake mechanisms in adrenergic nerves can be studied in a new way particularly by the iris technique developed by MALMFORS (1965), which makes possible adequate and direct studies on uptake in all parts of the postganglionic axons and terminals at both low and high CA concentrations. The preliminary experiments (HILLARP & MALMFORS 1964) have been greatly extended and show that the efficient uptake-concentration mechanism of the axon membrane is wholly insensitive to reserpine, but that the amines taken up after administration of this drug cannot be retained unless protected against destruction by the MAO present intraneuronally (MALMFORS 1965) However α-methyl-NA, which is readily taken up but not attacked by MAO accumulates within the non-terminal parts of the axons and in the terminals. Reserpine has also not been found to produce any increased leakage through the membrane. Even if administered NA (after MAO-inhibition) or α-methyl-NA has accumulated to high concentrations (in the order of 1,000–10,000 μg/g) without being bound to storage granules, since the non-terminal parts of the axons contain only few granules, the amines disappear only slowly over the course of many hours both in normal and reserpinized animals The adequacy of the methods used is shown by the fact that they readily permit demonstration of a blockade of the uptake mechanism in the membrane by several other drugs (such as desmethyl-imipramine, imipramine, chlorpromazine, cocaine) It seems impossible to inhibit MAO completely (at least *in vivo*) (CARLSSON 1960 WEISSBACH et al. 1961), and other paths for intraneuronal destruction of CA are likely to exist, although they are probably of minor physiological importance. Findings that smaller amounts of a *tracer* dose are retained in the tissue of reserpinized than of normal animals, even after pretreatment with a potent MAO inhibitor can therefore not be taken as evidence that reserpine acts on the cell membrane, e g by inhibiting an active transport into the adrenergic terminals.

It has recently been shown by *in vivo* (FUXE & HILLARP 1964) and more especially by *in vitro* experiments (HAMBERGER & MASUOKA, unpublished observation) that the adrenergic neurons in the brain also have a specific mechanism for uptake-concentration of CA, which operates efficiently even after the administration of reserpine.

The non-indole tetrabenazine – in contrast to reserpine – is a reversible, short-acting blocker of storage function (see PLETSCHER, BROSSI & GEY

1962) but there is good evidence that both have similar modes of action on the monoamine stores in the brain. They are both potent inhibitors of the specific Mg-ATP-dependent uptake mechanism of the medullary granules (CARLSSON HILLARP & WALDECK 1962, 1963) Experiments *in vivo* indicate that they compete for the same vital sites (QUINN, SHORE & BRODIE 1959 CARLSSON 1964c) and their effects on brain monoamines can be counteracted by pretreatment with MAO inhibitors. In our work *the two drugs have been found to produce the same rapid and complete* depletion of the amines stored in cell bodies and in synaptic terminals of the central adrenergic neurons. Both parts of the neurons recovered rapidly after tetrabenazine, whereas only the cell bodies showed a rapid recovery after reserpine The observations cannot be explained by the assumption that the drugs cause depletion primarily through action on the cell membrane. It must then be further postulated that reserpine has only a short-acting effect on the membrane of the cell bodies. The findings all fit, and indeed strongly support, the view that reserpine and tetrabenazine in this respect act by producing, respectively a long- and a short *acting block of the storage granules and that these* granules are continuously formed in the cell bodies (see below) Only this view seems, in fact, to afford a reasonable explanation for the findings mentioned below

1) After the administration of reserpine, the terminals showed a prolonged depletion for several days, and the cell bodies recovered more or less within 12 or 24 hours (see fig. 7) This phenomenon has been observed in all the monoaminergic neurons studied in this laboratory *e g* the peripheral adrenergic neuron (NORBERG & HAMBERGER 1964), and also the short and small DA neurons in the retina, which show an unusually rapid depletion (HÄGGENDAL & MALMFORS 1965 MALMFORS, unpublished results)

2) The cell bodies recovered at markedly different rates among the various cell groups in the cells showing rapid recovery after depletion induced by reserpine, the newly formed amines accumulated first in a zone around the nucleus The recovery phase after tetrabenazine was found to be entirely different new amines accumulated rapidly throughout the cell body and simultaneously in all the cells. In the former instance the depleted granules are blocked for several days and recovery is represented by the formation of new granules at different rates. In the latter the depleted granules are blocked only for a short time and then rapidly regain their storage capacity

There is now little doubt that the depletion of the amines in central and peripheral adrenergic neurons after administration of the DOPA

analogues *m*-tyrosine, α MMT or α-methyl-DOPA is produced by their corresponding amines, formed by intraneuronal decarboxylation, which displace the endogenous CA in the storage granules (CARLSSON & LINDQVIST 1962 ANDÉN 1964b CARLSSON 1964b CARLSSON *et al* 1964 CROUT *et al* 1964 SCHÜMANN & GROBECKER 1964) The depletion due to the amines formed from *m*-tyrosine is short lasting, probably because the amines can be readily displaced by newly synthesized CA and are readily attacked by MAO The amines formed from the α methylated amino acids in NA neurons, however are subsequently oxidized to the corresponding β-hydroxylated amines (aramine and α methyl NA), which for a long time block the storage of NA in the granules The DOPA analogues are thus excellent tools for studying the formation of these specific granules in NA neurons.

It was found in our work that both the cell bodies and the terminals of the adrenergic neurons were rapidly depleted after administration of meta-tyrosine and α-MMT After meta-tyrosine they both recovered rapidly and simultaneously After α MMT on the other hand, the NA terminals showed a long lasting depletion, but the cell bodies recovered at about the same rapid rate as after depletion induced by reserpine Here too, recovery could be seen in several instances to begin with an accumulation of amines in a zone around the nucleus. These findings furnish convincing evidence that the storage granules are formed in the cell bodies and strongly support the interpretation given above for the results obtained in the experiments with reserpine and tetrabenazine.

There is now almost conclusive evidence for the axoplasma flow theory of WEISS and that the protein synthesis of neurons can occur exclusively in the cell bodies (and possibly in dendrites) but not in the axons (for references, see DAHLSTRÖM 1965). It has further been shown that complex cellular structures, such as the zymogen granules in the pancreatic exocrine cells, are formed in the Golgi region (CARO & PALADE 1964). It therefore seems most improbable that the amine granules, which are complex structures *containing, e g., specific macromolecules, such as* enzymes, can be manufactured in any other part of the neuron besides the cell body

The findings in our work are in perfect agreement with this view Of great significance is the fact that newly formed amines, especially in the neurons showing rapid recovery after reserpine-induced depletion of the granules, appear first in a zone around the nucleus, where they can accumulate to high concentrations, then fill up the entire cell body and finally appear in the processes. There is little doubt that this represents the formation of new granules To the evidence discussed above it may

added that it seems improbable that CA can accumulate intraneuronally outside the storage granules to any great extent unless MAO is effectively inhibited (FUXE & HILLARP 1964 HAMBERGER *et al* 1964 HILLARP & MALMFORS 1964 MALMFORS 1965 NORBERG & HAMBERGER 1964). Studies on the formation and transport of the amine granules by constricting the axons of monoaminergic neurons (DAHLSTRÖM & FUXE 1964b & c DAHLSTRÖM 1965) strongly support the interpretations given above. They have furnished almost conclusive evidence that the granules are manufactured in the cell-bodies at a high rate, which varies greatly however from neuron to neuron, and are then transported down to the terminals This is strongly supported by other findings (DAHLSTRÖM, unpublished observation). Huge numbers of amine-containing granules accumulate rapidly in peripheral adrenergic axons above a constriction and to a lesser extent also below it owing to retrograde flow If the constriction is made simultaneously with the administration of a large dose of reserpine, however only the proximal accumulation develops. This occurs somewhat later at a time when the cell bodies just begin to show recovery but 2–3 days before the first sign of recovery can be detected in the terminals. This furnishes independent evidence that reserpine does not act primarily on the cell membrane From the results obtained in this and similar experiments, it can be concluded also that the drug can act directly on the granules and thus block the mechanism of their storage.

As shown in this and previous papers (DAHLSTRÖM & FUXE 1964a BARTONIČEK, DAHLSTRÖM & FUXE 1964), a marked and long lasting increase in 5-HT levels is rapidly obtained in the cell bodies, axons and terminals of the central 5-HT neurons after administering various potent but chemically different MAO inhibitors. This effect seems to be due to an inhibition of MAO (cf SHORE 1962) Since in the non-terminal parts of the axons there are normally only low concentrations of the amine, it seems safe to assume that they by analogy with the axons belonging to the peripheral adrenergic neurons (NORBERG & HAMBERGER 1964), contain only few storage granules. The amines that accumulate to high concentrations in this part of the neuron thus to a large extent must exist outside the storage granules If reserpine acts primarily on the cell membrane, it would consequently be expected that the drug will counteract the accumulation of amines occurring after administration of an MAO inhibitor It was, however found that not even a large dose of reserpine given shortly before a small dose of nialamide produced any obvious inhibition of 5-HT accumulation in the axons or cell bodies. This and the other results obtained all argue against the view that the cell membrane is the primary site of reserpine action.

Summary

The depletion and recovery of the amines stored in the cell bodies and synaptic terminals of the central adrenergic neurons in mouse and rat were studied after administration of reserpine and other short or long-acting depleters of neuronal catecholamines (tetrabenazine, *m*-tyrosine, α-methyl-meta-tyrosine) The highly sensitive fluorescence method used permits direct observations on the content and intraneuronal distribution of the amines

Both the cell bodies and the terminals showed about the same rapid depletion. After tetrabenazine and *m*-tyrosine, recovery was rapid and occurred simultaneously in both parts of the neurons. After reserpine, the terminals were depleted for several days, whereas the cell bodies recovered rapidly although at different rates, and often showed accumulations of newly formed amines, first in a zone around the nucleus The recovery of the noradrenaline neurons was similar after a depletion induced by α methyl-*m*-tyrosine. On the basis of these findings and other results obtained in direct studies on the uptake mechanisms and formation of the storage granules in adrenergic neurons, two conclusions are drawn. 1 The storage granules are formed in the cell bodies and then transported down to the terminals. 2. The primary action of reserpine in producing a long lasting block of storage function is not to block the mechanism for amine uptake (reabsorption) localized in the cell membrane of the entire adrenergic neuron, but to block the storage mechanism in the amine granules. The finding that 5-hydroxytryptamine readily accumulated to high concentrations within the cell bodies and axons of the hydroxytrypta minergic neurons in both normal and reserpinised animals after administration of nialamide further supports the view that the cell membrane is not the primary site of action of reserpine

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Mechanism of Amine Transport in the Cell Membranes of the Adrenergic Nerves

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Uptake by adrenergic neurons is an important factor in the inactivation of noradrenaline (NA). From data on selectively blocking agents it has been postulated that the uptake mechanism consists of two major components, active transport through the nerve cell membrane and a subsequent incorporation in the storage granule complex (CARLSSON HILLARP & WALDECK 1963) The latter step is efficiently blocked by reserpine and prenylamine (segontin ®) (see also KIRSHNER 1962 CARLSSON, HILLARP & WALDECK 1962) Cocaine, chlorpromazine and imipramine have previously been shown to block the uptake of NA, but they do not release NA already taken up (HERTTING, AXELROD & PATRICK 1961 AXELROD HERTTING & POTTER 1962) Recently it was demonstrated by histochemical techniques (HILLARP & MALMFORS 1964 and unpublished results) that these drugs and desmethylimipramine (DMI) act by blocking the active transport of amines through the nerve-cell membrane.

The aim of the investigation described here was to elucidate further and quantify the role of this transport mechanism

Methods

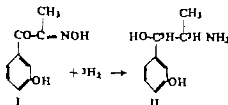
Mice weighing about 20 g, divided into groups of six were used throughout All injections were made intravenously unless otherwise stated Tissue extraction, ion-exchange chromatography of the extracts and subsequent liquid scintillation counting was performed essentially as described earlier (CARLSSON & WALDECK 1963a). Extracts from animals treated with ^{14}C-tyramine (TA) were adjusted to pH 6.0, others to pH 6.5 The elution procedure is indicated in table I A Tri-Carb liquid scintillation spectrometer was used for determining ^{3}H and ^{14}C (commercially available

Table 1

Elution procedure for Dowex 50 W X 4 200–400 mesh ion-exchange column diam. 4.2 mm height at pH 0 100 mm.

Eluant N HCl	Fraction
8 ml	Discarded
9–17 ml	Noradrenaline
10 ml	Discarded
11–30 ml	Metaraminol
12 ml	Discarded
13–26 ml	Octopamine

adrenaline and ^{14}C tyramine were used. ^3H-metaraminol (II) was prepared by catalytic tritiation of m-hydroxy-α-oximinopropiophenone (I) with palladium on charcoal.



Results

Mice were injected with ^3H-metaraminol and killed at various intervals. After ½ hr the level of ^3H-metaraminol was about ten times higher in heart than in femoral muscle. The ^3H-labelled amine was found to remain in the tissues for a long time (fig. 1). In the heart a continuous slow drop in activity was observed from about ½ hr throughout the rest of the experiment. In femoral muscle a proportionately more rapid decrease was observed between ½ and 3 hrs. In animals pretreated with reserpine the ^3H metaraminol was still taken up, but disappeared more rapidly from both tissues. Pretreatment with DMI caused an efficient block of ^3H metaraminol uptake by heart but not by femoral muscle. However the ^3H metaraminol taken up by the latter disappeared rapidly.

In another experiment mice were injected with ^3H NA and DMI was administered 15 min. later. After another 5 min. reserpine, metaraminol or α methyl metatyrosine (α MMT) was given. One hour after the administration of ^3H NA the animals were killed. Control groups not receiving DMI were run in parallel. Given alone DMI had no significant effect on the ^3H NA level, but completely blocked the release of ^3H NA

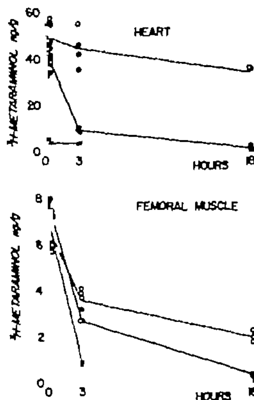


Fig. 1 Effects of reserpine and desmethylimipramine (DMI) on ^{3}H-metaraminol uptake and disappearance from mouse heart and femoral muscle.
o——o Control
●——● Reserpine
DMI
Reserpine, 10 mg/kg intraperitoneally was given 6 hrs. or DMI 10 mg/kg intravenously 5 min. before administering 0.02 mg/kg ^{3}H-metaraminol.

induced by metaraminol (fig. 2) In contrast, the reserpine induced release was practically unaffected by DMI. The release caused by α MMT was also blocked, though possibly not as effectively as the metaraminol induced release.

A similar experiment was performed to test the effect of DMI on the release of octopamine (OA) from the nerve endings (fig. 3). ^{14}C TA, which is rapidly converted to ^{14}C-OA in the tissues (CARLSSON & WALDECK 1963b), was injected into mice. After 15 min. when the ^{14}C-OA level was optimal and most of the ^{14}C TA had disappeared, DMI was given. After another 5 min. reserpine, metaraminol prenylamine or guanethidine was injected. The animals were killed 15 min later. Reserpine, metaraminol, prenylamine and guanethidine were used in doses that normally cause 90 % or more of the OA to disappear within 15 min.

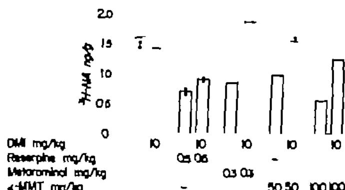


Fig. 2. Effect of desmethylimipramine (DMI) on drug-induced release of ³H-noradrenaline (NA) in the mouse heart.
³H NA 1 μg/kg was given intravenously and DMI 10 mg/kg 15 min. later. After another 5 min. reserpine 0.5 mg/kg, metaraminol 1 μM/kg (corresponding to 317 μg/kg of the bitartrate) or α methylmetatyrosine (α-MMT) 50 or 100 mg/kg was given by the same route. One hour after administering ³H NA the animals were killed. Controls not receiving DMI were run in parallel.

release induced by metarammol and guanethidine was completely inhibited by DMI whereas reserpine and prenylamme, despite DMI treatment, reduced the ^{14}C-OA content to less than 10 / of that in the control DMI given alone caused no significant reduction in ^{14}C-OA level.

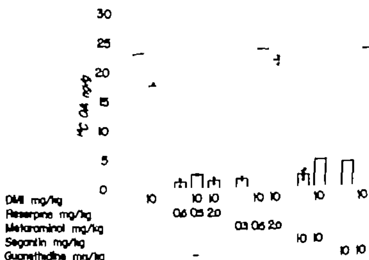


Fig. 3 Effect of desmethylimipramine (DMI) on drug-induced release of ¹⁴C-octopamine (OA) in the mouse heart.
¹⁴C-tyramine (TA) 0.2 mg/kg was given intravenously and DMI 10 mg/kg 15 min. later After another 5 min reserpine 0.5 or 2 mg/kg, metaraminol 0.5 or 2 mg/kg, segontin ® (prenylamine) 10 mg/kg or guanethidine 10 mg/kg was given by the same route. The animals were killed 35 min. after the injection of ¹⁴C-TA. The controls not receiving DMI differed in that the releasing drug was given 15 min. after the injection of ¹⁴C TA. The animals were killed after another 15 min. Further the controls receiving metaraminol were given 1 μM/kg corresponding to 317 μg/kg of the bitartrate.

Discussion

Metaraminol, a sympathomimetic amine resistent against monoamine oxidase, as well as catechol-O-methyl transferase, appears to have a high affinity for the granular storage sites (ANDÉN 1964 CARLSSON & WALDECK, unpublished results). This may explain why the heart, which is richer in adrenergic nerves and NA and has a higher capacity to take up NA, took up more metaraminol and retained it more efficiently than did femoral muscle In fact, experiments in this laboratory have shown that the uptake of metaraminol by a tissue rich in NA (submaxillary gland) is markedly reduced after chronic sympathetic denervation (ALMGREN & WALDECK, unpublished results). The more rapid initial loss of ^{3}H metaraminol in femoral muscle in the experiments here recorded may indicate a higher percentage of extraneuronal binding. When the granule storage mechanism was impaired by reserpine, the ^{3}H metaraminol taken up by the neuron through the action of the "cell-membrane pump" disappeared more rapidly – *probably by leakage through the cell membrane – as it could not be incorporated in the granules*. On the other hand, when the transport through the nervous cell membrane was inhibited by DMI, the uptake of injected ^{3}H metaraminol by the heart was efficiently blocked. In the femoral muscle, which probably has a high proportion of extraneuronal binding, DMI had no significant effect initially Since femoral muscle contains adrenergic nerves, *a certain difference might have been expected*. However this may have been masked by a genuine increase in the concentration of metaraminol in the interstitial fluid and on extraneuronal binding sites (cf ALMGREN ANDÉN & WALDECK 1965), an inevitable consequence of the general blockade of the adrenergic "cell membrane pump"

The experiments on release of ^{3}H NA and ^{14}C-OA reveal a marked difference between reserpine and metaraminol. Whereas the effect of reserpine was unaffected by DMI amine release by metaraminol was inhibited. In the OA experiments guanethidine behaved in the same way as metaraminol and prenylamine as reserpine. The results may be explained by the fact that lipophilic compounds, such as reserpine and prenylamine, are able to penetrate biological membranes without the aid of active transport mechanisms. On the other hand, hydrophilic amines, such as metaraminol and guanethidine, are less able to do so and thus depend to a higher degree on transport and other mechanisms facilitating penetration. Consequently the penetration of metaraminol and guanethidine can be blocked by DMI, whereas the permeation of reserpine and prenylamine is unaffected by DMI – The results of STONE *et al.* (1964), which are in good agreement with our observations, may be explained accordingly

The fact that the amine releasing action, not only of metaraminol but also of α-MMT was blocked by DMI indicates that the decarboxylation of α-MMT which must precede the release (CARLSSON & LINDQVIST 1962), has taken place mainly extraneuronally. It may be recalled that certain tissues, e. g. the kidney are extremely rich in extraneuronal DOPA decarboxylase (HOLTZ, HEISE & LÜDTKE 1938).

In the dose employed (10 mg/kg intravenously) guanethidine produced a transient sympathomimetic response (piloerection, exophtalmus, etc.). In the animals pretreated with DMI guanethidine failed to produce this response. It would thus appear that guanethidine has to be transported through the cell membrane of the adrenergic nerve terminal in order to produce its sympathomimetic response. It may be recalled that this response is also blocked by reserpine pretreatment (see SHORE 1960). It may thus be concluded that the sympathomimetic activity of guanethidine is brought about by release of NA from a pool located inside the cell membrane of the adrenergic nerve terminal, probably in the adrenergic storage granules.

KUNTZMAN *et al* (1962) have pointed out that the release of NA from adrenergic nerve terminals by guanethidine and reserpine must be assumed to be produced by different mechanisms. The release by the former drug is accompanied by signs of sympathomimetic activity indicating release "onto receptor sites" analogously with the nerve impulse. The release by the latter drug is due to impairment of the storage mechanisms and occurs "onto MAO". In the light of our experiments, the difference between guanethidine and reserpine may be explained at least partly by the blocking action of the former drug on amine transport at the cell membrane. The results described show that guanethidine utilizes this transport mechanism. Histochemical observations (MALMFORS, unpublished results) and unpublished results in this laboratory show that such utilization, as might be expected, is accompanied by a blocking action on transport. Consequently the NA release induced by guanethidine cannot be neutralized by the action of the membrane pump as with reserpine. If this is the correct explanation, reserpine should produce a sympathomimetic response when given to animals pretreated with DMI. When the experiments represented by fig. 3 were performed, it was noted that reserpine, given to animals pretreated with DMI caused a sympathomimetic response just as guanethidine did when given alone. Thus an explanation is offered for the paradoxal phenomenon that DMI blocks the sympathomimetic activity of guanethidine but provokes a sympathomimetic activity of reserpine.

However this may not explain all the differences between guanethidine and reserpine in pharmacological activity at the adrenergic nerve termi-

nals. As is well known, the transmission block induced by the former drug, in contrast to the latter sets in fairly quickly and long before the transmitter store is depleted (see MUSCHOLL 1964). Thus guanethidine, in contrast to reserpine, must be assumed to have a direct inhibitory influence on the mechanism that causes the adrenergic transmitter to be released by the nerve impulse. Also this action of guanethidine may be prevented by DMI and similar agents (STONE *et al.* 1964) indicating that guanethidine has to pass the nerve-cell membrane to produce its effect.

Summary

^{3}H-metaraminol was given to mice, and the uptake and disappearence of the amine in heart and skeletal muscle were studied. After pretreatment with reserpine, ^{3}H metaraminol was still taken up but was lost more rapidly presumably owing to the blocking action of the drug on the storage function of the nerve granules. Desmethylimipramine (DMI) completely blocked the uptake of ^{3}H-metaraminol, presumably owing to inhibition of the amine-transport mechanism of the cell membrane of the adrenergic nerves. An examination of the effect of DMI on the release of ^{3}H-noradrenaline and ^{14}C-octopamine caused by different drugs revealed that hydrophilic compounds, such as metaraminol and guanethidine depend on the active transport mechanism through the nerve cell membrane, whereas lipophilic compounds, such as reserpine and prenylamine, did not depend on this mechanism for their penetration into the adrenergic nerves.

The results support the view that two different amine-concentrating mechanisms exist in the adrenergic nerves, one located in the storage granules and inhibited by reserpine, the other located in the cell membrane and inhibited by DMI. Against this background, several hitherto obscure drug effects on adrenergic mechanisms may be explained.

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Effects of Hydrocortisone, Thyroxine, Reserpine and Monoamine Oxidase Inhibitors on the Blood Glucose of Rats under Diethyl Ether Anaesthesia

By

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Diethyl ether anaesthesia increases the blood glucose content (WYMER 1926) through the adrenals, because in adrenalectomized animals there is no rise in blood glucose (PHILLIPS & FREEMAN 1933) Under ether anaesthesia the amount of catecholamines increases in human peripheral blood, which partly explains the rise in blood glucose (PRICE *et al.* 1959) During ether anaesthesia of the rat the rise in blood glucose is rapid and of short duration (MANNINEN *et al* 1965) but in man it is continuous and long lasting (HENNEMAN *et al.* 1961)

Corticoids increase the glycogen reserves in the liver and blood glucose content (BRITTON & SILVETTE 1932 1934) owing to increased gluconeogenesis (BERGENSTAL *et al.* 1952 ALBANESE *et al* 1954 HAYNES 1961) and decreased peripheral utilization of carbohydrates (SAYERS *et al* 1949 FORSHAM *et al* 1950 HAUSBERGER *et al* 1955).

Thyroxine speeds up the utilization of glucose by tissues (MIRSKY *et al* 1936) and depletes the liver glycogen stores (STERNHEIMER 1936) Thyroxine also increases glycogenolytic activity in the liver of rats as shown by isotope (^{14}C) technique (GLOCK *et al* 1956) Thyroxine has a direct glycogenolytic action in the liver Its glycogenetic action is probably mediated through an increased adrenal secretion of 17-hydroxy 11 hydroxysteroids its glycogenetic action may interfere or mask its glycogenolytic action (TIMIRAS *et al* 1955).

After a large dose of reserpine (5 mg/kg I.V.) the catecholamines disappear almost completely from the brain, heart and adrenals of the rabbit within 1,3 and 16 hours (CARLSSON *et al.* 1957) and also clearly after a small dose (100 µg/kg) in several tissue of the rat (PEKKARINEN *et al*

1958) it increases excretion of adrenaline into the urine twenty fold, but only doubles the noradrenaline content (HAZARD *et al* 1960 BICKEL *et al.* 1961) The released adrenaline and noradrenaline are immediately metabolized (HAZARD *et al* 1960) Reserpine (in doses 0.1–1 0 mg/kg) increases the secretion of ACTH and accelerates the gluconeogenesis (BALZER & PALM 1962) It increases the glycogen in rat s liver (with 268 %), in sceletal muscle (111 %) in heart muscle (174 %) and in the brain (71 %) (BALZER & PALM 1962) and raises the blood glucose (5–158 %) (BALZER & PALM 1962) In adrenalectomized animals this effect is absent (KUSHKE & FRANZ 1955 HOLZ *et al* 1957). A temporary depletion of adrenaline and noradrenaline reserves in the adrenals has been brought about by doses of 2 mg/kg over 3 days (ERÄNKÖ & HOPSU 1961) The monoamine oxidase inhibitors, on the other hand, protect noradrenaline from enzymatic deamination (IISALO 1962) If 50 mg/kg of iproniazid is administered, it increases the noradrenaline content of rat s tissues (36–79 %) in 6–10 hours (PEKKARINEN *et al* 1958) After a single injection of iproniazid a marked increase in catecholamines, depending in amount on the dose of iproniazid, has been observed in the tissues of experimental animals (PLETSCHER *et al* 1957), and pretreatment with iproniazid was found to prevent to a marked extent the depleting effect of reserpine on catecholamines in the tissues (CARLSSON *et al.* 1957 PLETSCHER 1957 ZBINDEN 1958 PEKKARINEN *et al* 1958) Iproniazid also prevents the rise of glycogen content in the liver after reserpine (HOLZ *et al.* 1957)

The adrenergic system, and above all adrenaline secretion by the adrenals, is the most important regulator of blood glucose. The purpose of this investigation is to make clear how hormones, thyroxine and hydrocortisone, as well as the drugs that either liberate adrenaline or noradrenaline or cause their accumulation, e. g. reserpine or MAOI, alters the blood glucose content during ether anaesthesia.

Material and Methods

The *drugs and chemicals* used were Hydrocortisone (scheroson F ®, Schering), iproniazid (marsilid ®, La Roche), isocarboxazid (marplan ®, La Roche), thyroxine (tyroxin ® Star), reserpine (serpasil ®, Ciba), diethyl ether (Aether ad narcosin ®, Orion), sodium tungstate (The British Drug Houses Ltd), sulphuric acid (Merck, Sharp & Dohme), 2-aminobiphenyl (L. Light & Co), and glacial acetic acid (Merck, Sharp & Dohme).

The *experimental animals* were pretreated for 4 days with drugs administered as shown below

Thyroxine	0.005	mg/kg/day	oral
Reserpine	1.0	–	i. m.
Iproniazid	100.0	–	i. p.
Isocarboxazid	100.0	–	oral
Hydrocortisone	5.0	–	oral

For the ether anaesthesia an open mask was used into this was dropped ethyl ether as needed. The ether concentration of the inspired air cannot be evaluated in the open mask procedure, and the drugs modified greatly the ether anaesthesia and the need of ether in amount sufficient for anaesthesia. The experimental animals were kept in such deep anaesthesia that the corneal reflex was abolished.

The animals included 87 male white rats (270–450 g) pretreated 4 days before the test. On the fifth day the first blood sample was taken from the tail vein without anaesthesia and then at certain intervals during the 1.5–2.5 hours continuous anaesthesia for determining the blood glucose.

Proteins were precipitated by the Folin and Wu method (FOLIN & WU 1920) from 0.1 ml blood. The samples were centrifuged 5 min. later. The determination of blood glucose (ATHANAIL & CORALLO 1958, FORSELL & PALVA 1959) was modified. 0.5 ml of clear filtrate was added to 2.5 ml of 0.4% (w/v) 2-aminobiphenyl solution. The green colour was developed by boiling under 2.27 atm. pressure for 20 min., and the extinction was measured in a Beckman B spectrophotometer 6500 10^{-10} m wavelength.

The standard curve for colour reaction and the standard curve for the method were linear up to 1.0 mg/ml, but for higher values they deviated, as fig. 1 a shows, though only slightly. From 2.0 mg/ml upwards the curves were again linear.

Results

Ether anaesthesia without drugs, (19 rats) figs. 1 b. Before the beginning of the anaesthesia the blood glucose content was 1.05 ± 0.04 mg/ml ($n = 19$). 30 min. after the beginning of anaesthesia it had risen to its maximum, 1.34 ± 0.06 mg/ml ($n = 19$), and after 120 and 150 min. it was almost at the starting value. From then on the blood glucose decreased continuously. The increase in blood glucose also became less uniform at the end of the test. The difference between maximal and minimal blood glucose contents 1.04 ± 0.04 mg/ml ($n = 19$) and 1.34 ± 0.06 mg/ml ($n = 19$) was significant ($p < 0.001$).

Hydrocortisone + ether anaesthesia, (13 rats) fig. 1c. The blood glucose content was 1.04 ± 0.03 ($n = 12$) at the beginning of the anaesthesia, from which it rose until the end of the anaesthesia. After 90 min. it was 1.50 ± 0.12 mg/ml ($n = 13$). The increase in blood glucose content was uniform in the animals of this group, with a few exceptions. The difference between the maximal and minimal blood glucose contents, 1.04 ± 0.03 mg/ml ($n = 13$) 1.50 ± 0.12 mg/ml ($n = 13$) was significant ($p < 0.01$).

Thyroxine + ether anaesthesia, (10 rats), fig. 1d. The blood glucose content at the beginning of the test was 1.07 ± 0.05 mg/ml ($n = 10$). It rose as long as anaesthesia lasted. After 60 min. the content rose to its maximum 1.55 ± 0.07 mg/ml ($n = 10$). The increase in blood glucose was fairly uniform at the beginning of the test, and dispersion appeared after 60 min. of anaesthesia. The difference between maximal and minimal

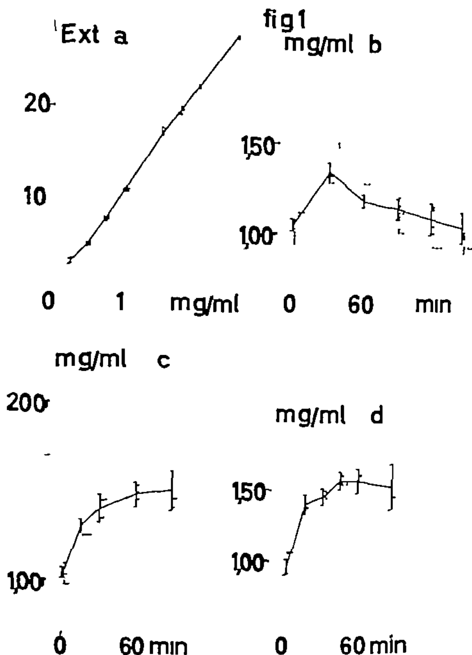


Fig. 1

a. The colour reaction curve for glucose.
b. Blood glucose content in ethyl ether anaesthesia and
c. after 4 days hydrocortisone (5 mg/kg p.o.) pretreatment.
d. after 4 days thyroxine (5 µg/kg p.o.) pretreatment.

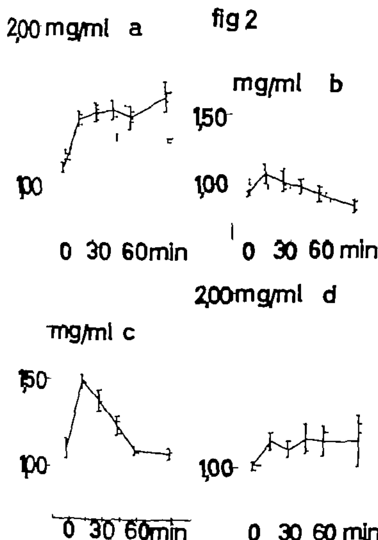


Fig. 2. Blood glucose content in ethyl ether anaesthesia
a. After 4 days thyroxine (5 µg/kg p.o.) and reserpine (1 mg/kg i.m.) pretreatment.
b. After 4 days iproniazid (100 mg/kg i.p.) pretreatment.
c. after 4 days iproniazid (100 mg/kg i.p.) and hydrocortisone (5 mg/kg p.o.) pretreatment.
d. After 4 days isocarboxazid (100 mg/kg p.o.) pretreatment.

blood glucose contents, 1.07 ± 0.05 mg/ml ($n = 10$) and 1.55 ± 0.07 mg/ml ($n = 10$), was significant ($p < 0.001$)

Thyroxine + reserpine + ether anaesthesia, (16 rats), fig. 2a. The basal blood glucose, 1.11 ± 0.03 mg/ml ($n = 16$), rose steeply during the whole period of anaesthesia 15 min. after the beginning of anaesthesia it was 1.38 ± 0.04 mg/ml ($n = 16$) and at 90 min. at its maximum, 1.50 ± 0.09 mg/ml ($n = 13$) The blood glucose increase was uniform, although

dispersion appeared. The rats resisted the test better than after reserpine only. The difference between maximal and minimal blood glucose contents 1.11 ± 0.03 mg/ml (n = 16) and 1.50 ± 0.09 mg/ml (n = 13), was significant ($p < 0.001$).

MAO inhibitors

Iproniazid + ethyl ether anaesthesia, (11 rats), fig. 2b. The basal blood glucose content was 0.95 ± 0.02 mg/ml (n = 11), from which it rose in 15 min. during ether anaesthesia to its maximum of only 1.06 ± 0.06 (n = 11). After 90 min. it was only 0.87 ± 0.04 mg/ml (n = 10) below the starting level. No clearly rising curve appeared on the basis of these results. The difference between maximal and minimal blood glucose contents was not significant.

Hydrocortisone + iproniazid + ether anaesthesia, (9 rats), fig. 2c. The basal blood glucose content was 1.11 ± 0.06 mg/ml (n = 8). In 15 min. during anaesthesia the blood glucose rapidly reached its maximum 1.50 ± 0.04 mg/ml (n = 9) from which it decreased evenly until the end of the test, being 1.09 ± 0.03 mg/ml (n = 7) at 90 min. The final value was thus below the basal level. The blood glucose increase was uniform, and the rats resisted the test well. The difference between maximal and minimal blood glucose contents was significant ($p < 0.001$).

Isocarboxazid + ether anaesthesia, (9 rats) fig. 2d. The basal value of the blood glucose content was 1.01 ± 0.02 mg/ml (n = 9) and rose in 15 min. during the ether anaesthesia to 1.15 ± 0.05 mg/ml (n = 9), at which level it remained until the end of the test. No clearly rising curve appeared in this group either. The response of the blood glucose content was uneven: three high values were found in the same test animal. The difference between maximal and minimal blood glucose contents was significant ($p < 0.05$).

Discussion

During ether anaesthesia without drugs the rise in rats' blood glucose content was rapid and of short duration (MANNINEN *et al.* 1965). This was, however, not due to any depletion of glycogen reserves, but to the fact that the ether stimulus seems to be too weak to maintain a sufficient strong adrenaline secretion from the adrenal medulla (MANNINEN *et al.* 1965). In rats pretreated with hydrocortisone the blood glucose rose during ether anaesthesia to a remarkably high level and remained there for a long time. This shows that the hydrocortisone improves the glycogen reserves of the organism (BRITTON & SILVETTE 1932, 1934).

Thyroxine increases the basal and carbohydrate metabolic rate (MIRSKY *et al* 1936) and depletes the glycogen reserves of the liver of test animals (GOODESHALL *et al* 1933 STERNHEIMER 1936). Depending on the dose, it may stimulate the adrenal cortex and improve gluconeogenesis through the action of the adrenals (TIMIRAS *et al* 1955) Owing to this mechanism, the glycogen reserve of the organism can remain and stand up to a con sumption greater than normal. In our investigations the blood glucose increased at the beginning of anaesthesia to 1.35 mg/ml and remained at this level or slightly higher until the end of the anaesthesia. A slight tendency of blood glucose to decrease during anaesthesia can be considered as a normal biological change

In the group pretreated simultaneously with thyroxine and reserpine the blood glucose showed a rise to 1.50 $\pm$ 0 09 mg/ml (n = 13) in 90 min. of ether anaesthesia. This shows clearly the rapid increase in blood glucose in the presence of thyroxine and reserpine, in spite of the raised consumption of glycogen caused by thyroxine Reserpine alone, on the other hand did not bring about any rapid rise in blood glucose, but caused a slow continuous increase during ether anaesthesia (MANNINEN *et al* 1965)

It was interesting to observe the weakening effect of the MAO-inhibitors iproniazide and isocarboxazid on the rise in blood glucose during ether anaesthesia, consistent with the earlier finding that iproniazid decreases liver glycogen content (HOLZ *et al* 1957) In the rats treated with ipronia zid the increase in blood glucose during anaesthesia was small, only 0.11 mg/ml above the basal level, and showed a continuous decrease at the end of the test. These results could indicate a depletion of liver glycogen reserves by pretreatment with iproniazid The increase in adrena line and noradrenaline contents of the peripheral tissues caused by iproniazid might also lead to a mobilization of glycogen from the liver Another explanation may be that iproniazid inhibits the secretion of adrenaline or noradrenaline from the adrenergic nerve endings or from adrenal medulla during ether anaesthesia, as indicated clinically by the hypotension, especially in hypertensive patients, occurring during iproniazid treatment.

The simultaneous pretreatment with iproniazid and hydrocortisone caused a rapid large increase in the blood glucose content during anaesthesia. The rapid rise in blood glucose leads to an equally rapid decrease, as also seen in ether anaesthesia without pretreatment with drugs, from which we can conclude that the glycogen reserves in the liver had been depleted or that the adrenergic stimulus is rapidly weakened during anaesthesia. Thus iproniazid has clearly modified the power of hydrocortisone continuously to increase blood glucose as well as liver glycogen reserves.

Summary

1 The object of this study was to clear up the effect of 4 days pretreatment with hydrocortisone, thyroxine, reserpine, iproniazid or isocarboxazid on the blood glucose in rats during diethyl ether anaesthesia. In the control group the ether anaesthesia caused a rapid and temporary rise in blood glucose content to 1.34 ± 0 06 mg/ml (n = 19), 0.29 mg/ml above the basal level.

2. Hydrocortisone pretreatment caused a large continuous rise in blood glucose during anaesthesia.

3 In rats pretreated with thyroxine there was also a large continuous increase in blood glucose.

4 Simultaneous pretreatment with thyroxine and reserpine showed clearly that thyroxine fastens the increase of blood glucose then also, compared with a slow increase in the group having reserpine pretreatment.

5 After MAO-inhibitors (iproniazid and isocarboxazid) the blood glucose did not increase clearly during ether anaesthesia. They reduce the normal initial rise in blood glucose during ether anaesthesia.

6 In the group given iproniazid and hydrocortisone at the same time, the increase in blood glucose was rapid but only brief in contrast to the hydrocortisone group showing a continuous blood glucose increase in ether anaesthesia.

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The Effect of Drugs Influencing the Sympathetic Nervous System upon Blood Glucose Regulation in Rats during Diethyl Ether Anaesthesia

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(Received November 10, 1964)

Diethyl ether anaesthesia increases the blood glucose (WYMER 1926) through the adrenals, since in adrenalectomised animals it does not cause any hyperglycaemia (PHILLIPS & FREEMAN 1933–34) In man ether anaesthesia causes an increase (0.57–1 17 mg/ml) in blood glucose lasting for several hours (HENNEMAN & BUNKER 1961) It also raises the nor adrenaline content of the peripheral blood (PRICE *et al* 1959)

Reserpine in large doses (0 1–1 0 mg/kg) increases the secretion of ACTH by which gluconeogenesis accelerates owing to the rise in glucocorticoid secretion (BALZER & PALM 1962) Reserpine increases the glycogen content of rats liver (with 268 %) of sceletal muscle (111 %), of heart muscle (174 %) and of the brain (71 %) (BALZER & PALM 1962) and the glucose content of the blood (5–158 %) In adrenalectomized animals this effect does not appear (KUSHKE & FRANZ 1955 HOLZ, BALZER & WESTERMANN 1957). A temporary depletion of adrenaline and noradrenaline in the adrenals has been brought about by doses of reserpine (2 mg/kg) over 3 days (ERÄNKÖ & HOPSU 1961) The regulation of blood glucose content after reserpine treatment is interesting, because reserpine is a noradrenaline liberator in the tissues and decreases the reflex regulation of circulation by the sympathetic nervous system (VARLEY & GOWENLOCK 1963 HIMWICH & HIMWICH 1964)

Inhibition of the secretory function of the sympathetic nervous system can be brought about by guanethidine and bretylium. They do not seem to affect the adrenal medulla for certain (BRODIE & KUNZMAN 1960 KRONEBERG *et al* 1962) Guanethidine blocks the transmission of the

nerve impulse in sympathetic nerve endings (MAXWELL *et al* 1960), decreases the noradrenaline content in the tissues and inhibits its release from the sympathetic postganglionic nerve fibres (RICHARDSON 1960)

Bretylium is a sympathetic inhibitor (CONWAY 1960) and can prevent the noradrenaline depletion caused by reserpine (INESI *et al* 1962 VARLEY & GOWENLOCK 1963 HIMWICH & HIMWICH 1964) Further it also inhibits the release of noradrenaline after guanethidine administration (COSTA 1961).

α-Methyldopa decreases the excretion of vanilmandelic acid in the urine (SCHAUB *et al.* 1962) This is due to the inhibition of the decarboxylase, which in its turn hinders the synthesis of the precursor of adrenaline and noradrenaline, dopamine.

The increase in glycogen reserves of the liver and the blood glucose caused by corticoids has been recognized for a long time (BRITTON & SILVETTE 1932 1934) This effect is due to increased gluconeogenesis (HAYNES 1961) Adrenaline and cortisone together cause a greater rise in blood glucose than adrenaline alone (VOLK & LAZARUS 1959).

The purpose of our study has been to investigate the effects of drugs that influence the adrenergic regulation of the blood glucose of rats during ether anaesthesia. Adrenaline secreted by the adrenal medulla is the important metabolic regulator of blood glucose content.

Material and Methods

The drugs

Ethyl ether (Aether ad narcosin ® Orion), adrenaline (Dr Theodor Schuchardt, GMBH & Co), lidocainechloride (xylocain ®, Astra) hydrocortisone (scheroson F ®, Schering Leiras) reserpine (serpasil ®, Ciba), bretyliumtosylate (darenthin ® Burroughs Wellcome & Co). Alphamethyldopa (aldomet ® Merck, Sharp & Dohme)

The reagents.

The reagents are presented in our preceding publication. (MANNINEN *et al.* 1965). To 154 male rats weighing 270–450 g were given the drugs during four days before the test. On the fifth day a control blood sample was taken from the tail vein the rats were then anaesthetized with ether by an open mask method. The anaesthesia was kept deep enough for the corneal reflex to be abolished. Blood samples were taken at intervals during the anaesthesia for determination of the glucose content.

The method of treating the samples has been described i the preceding publication.

Results

1 *The control group* ether anaesthesia without drugs (19 rats), fig. 1a, is the same as in a previous study and is shown for comparison (from MANNINEN *et al* 1965)

2 *Adrenaline + ether anaesthesia* (12 rats), fig. 1d. Adrenaline 1 mg/kg was injected s. c. into the rats 15 min. before taking the first blood

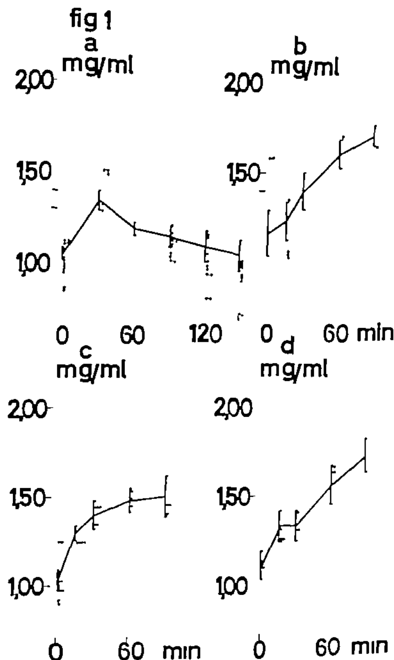


Fig. 1 The blood glucose content of rats

a. During diethyl ether anaesthesia (from MANNINEN *et al* 1965).
b. after adrenaline (1 mg/kg s.c.) and lidocain local anaesthesia.
c. during ether anaesthesia fter 4 days hydrocortisone pretreatment (5 mg/kg p.o.) (from MANNINEN *et al* 1965).
d during ether anaesthesia after drenaline injection (1 mg/kg s.c.).

The glucose content of the blood rose steeply during the whole period of ether anaesthesia, reaching in 90 min. as high a value as 1 72 ± 0 01 mg/ml which shows that liver glycogen is strongly mobilized by adrenaline. The difference between maximal and minimal blood glucose contents, 1 10 ± 0.09 and 1 72 ± 0.10 mg/ml, was significant ($p > 0.001$).

3. *Adrenaline + lidocain local anaesthesia* (12 rats), fig. 1b On administering adrenaline as described above and anaesthetizing locally the tail with 2 / xylocain ® the basal glucose content was 1 17 ± 0.13 mg/ml without ether anaesthesia. The glucose content also in this group rose steeply to a high value, being after 90 min 1 72 ± 0 06 mg/ml The dispersion in this group was great, but the difference between minimal and maximal blood glucose contents was significant ($p > 0.01$)

4. *Hydrocortisone + ether anaesthesia* (13 rats), fig. 1c. The curve is taken from a previous study (MANNINEN *et al* 1965) and is shown for comparison.

5. *Reserpine + ether anaesthesia* (18 rats), fig. 2a. When 1 mg/kg of reserpine was given i. m. over four days, the control value of the blood glucose content was clearly increased to 1.25 ± 0 05 mg/ml. The maximum in the control group of normal rats during the initial stage of ether anaesthesia did not occur in this group, but the rise in the blood glucose content appeared slowly and continued to increase slightly until the end of the anaesthesia. When the anaesthesia had lasted for 150 min. the blood sugar was as high as 1.58 ± 0 10 mg/ml. The total rise was 0.32 mg/ml during 2½ hours anaesthesia. The animals could not resist the prolonged anaesthesia well and showed strong bronchial secretion. The difference between minimal and maximal blood glucose contents was significant ($p > 0.001$)

6. *Reserpine + adrenaline + ether anaesthesia* (18 rats), fig. 2b When administering reserpine as in group 5 (above), along with 1 mg/kg of adrenaline s c. 15 min. before taking the first blood sample, the glucose content of the blood rose already to 1.35 ± 0 06 mg/ml. During the ether anaesthesia there was a marked and steep rise in blood glucose. After 90 min. it reached 1.88 ± 0 10 mg/ml and at 120 min. 2.15 mg/ml (only two samples were taken) In spite of the high basal blood glucose content its rise was marked. The difference between minimal and maximal blood glucose contents, 1.35 ± 0.06 and 1.88 ± 0 10 mg/ml, was significant ($p > 0.001$).

7 *Guanethidine + ether anaesthesia* (19 rats), fig. 2d. When 20 mg/kg guanethidine had been given for four days i. p. before the test, the blood glucose content was lower than in the control group, and its tendency to rise during ether anaesthesia was weak. No clear maximum appeared in blood glucose content.

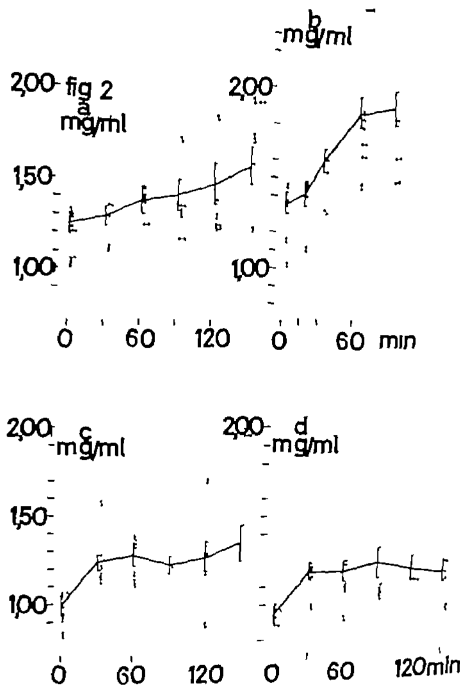


Fig. 2. Blood glucose content of rats during ether anaesthesia
a. after four days reserpine pretreatment (1 mg/kg i.m.).
b. fter four days reserpine pretreatment (1 mg/kg i.m.). and adrenaline injection (1 mg/kg s.c.).
c. after four days bretylium pretreatment (25 mg/kg p.o.)
d. after four days guanethidine pretreatment (20 mg/kg i.p.).

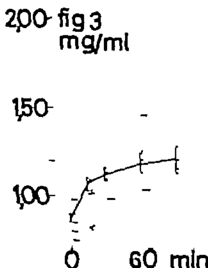


Fig. 3. The effect of α methyldopa (25 mg/kg p.o. four days) upon the blood glucose content of rats during ether anaesthesia.

8. *Bretylium + ether anaesthesia* (17 rats), fig. 2c. Similarly when bretylium was given 25 mg/kg p o over four days before the test, the basal value of the blood glucose content 0 99 ± 0 05 mg/ml was lower than in the control group It was 1.24 ± 0 04 mg/ml 30 min. after the anaesthesia had begun. In this group also there appeared an almost equally clear initial rise in the control group due to the basal content of the blood glucose content being stabilized nearly at this level The difference between initial and final values was nearly significant ($p > 0.05$).

9. *α Methyldopa + ether anaesthesia* (19 rats), fig. 3 After administration of α-methyldopa, 25 mg/kg for four days p o through a stomach tube, the basal blood glucose content 0 87 ± 0 02 mg/ml was clearly lower than in the control group Thus there was a clear initial rise, although not a very steep one. Half-an-hour after the beginning of the anaesthesia the blood glucose content was 1 12 ± 0 04 mg/ml. The reaction of this group was more uniform than that of the other groups. The difference between minimal 0 87 ± 0 02 and maximal blood glucose contents, (after 90 min.) 1.20 ± 0 08 mg/ml, was highly significant ($p < 0.001$)

10. *Adrenalectomy + reserpine + ether anaesthesia* (7 rats) Seven rats were adrenalectomized also 1 mg/kg reserpine was administered The ether anaesthesia did not cause any rise in the blood glucose content, which remained at the 0 90 mg/ml level throughout the anaesthesia. The rise in blood glucose, caused by ether anaesthesia, originates to a large extent from the adrenal medulla.

Discussion

The increasing effect of ether anaesthesia on blood glucose content appears in our experiments in the normal rats as temporary and swift, whereas in man it is continued and long lasting (HENNEMAN & BUNKE 1961) This may indicate a depletion of liver glycogen reserves and a weak liver glycogen mobilisation mechanism in the rat under ether anaesthesia, which, however combined with exogenous adrenaline injection, caused a steep continuous and more marked rise in blood glucose content than did ether anaesthesia alone thus the liver had a good glycogen reserve, but the ether anaesthesia was not a sufficient stimulus to bring about the continuous increase in glucose content as a result of a continuous secretion of adrenaline from the adrenal medulla. Obviously ether anaesthesia does not cause as great a secretion of adrenaline from the adrenal medulla as does exogenous adrenaline administration.

The effect of reserpine and guanethidine as adrenaline and noradrenaline liberators upon carbohydrate metabolism should also be noted, as should the blood glucose content during ether anaesthesia. Reserpine pretreatment increased the basal content of blood glucose in two groups of rats (1.25 and 1 35 mg/ml) to a higher level than that in the hydrocortisone group (1 04 ± 0.03 mg/ml) After reserpine pretreatment, ether anaesthesia brought about a continuous and slow rise in the blood glucose to 1.58 ± 0 10 mg/ml, as opposed to just the rapid initial increase in the control group The tendency of the blood glucose to rise after reserpine during the ether anaesthesia, although slow can be compared with that of hydrocortisone or adrenaline group, though they showed a fast initial rise The reserpine dose, 1 mg/kg during four days, was not sufficient for depletion of adrenaline, and the adrenal medulla still contained enough adrenaline to raise the blood glucose slowly during ether anaesthesia.

The effect of ether anaesthesia did not appear in adrenalectomized reserpine-treated rats, showing that the blood glucose increase in the reserpine group was also due to adrenaline secretion from the adrenal medulla. Adrenaline was still able to enhance the increase in blood glucose content reduced by reserpine to 1.35 ± 0 06 mg/ml, during ether anaesthesia to 1 88 ± 0 10 mg/ml

After guanethidine treatment the rise in basal content of blood glucose during ether anaesthesia to 1.23 ± 0 09 mg/ml was lower but more long-lasting than in the control group The retarded effect could depend on the effect of guanethidine which depletes the postganglionic sympathetic nerve ends from noradrenaline in the tissues. Distribution of blood glucose content shows a large scatter in this group, with several blood glucose values under 1 10 mg/ml.

Bretylium has a modifying effect on blood glucose content similar to that of guanethidine. The variation in distribution of blood glucose observed is like that in the guanethidine group.

α-Methyldopa decreases the adrenaline and noradrenaline contents of tissues by inhibiting the synthesis of the adrenaline and noradrenaline precursor dopamine, which leads to increased gluconeogenesis (KOBAYASHI 1960). In our experiments the control value of blood glucose after treatment with α-methyldopa was lower than in any other group 0.87 ± 0.02 mg/ml, but it increased clearly to 1.20 ± 0.08 mg/ml, showing a wide scatter of blood glucose values during ether anaesthesia.

After the antihypertensive drugs reserpine, quanethidine, bretylium or α-methyldopa, the increase in blood glucose continued during the whole of ether anaesthesia, wheres in untreated rats the increase in blood glucose content appears only in the initial phase of ether anaesthesia.

Summary

The effect of the newer antihypertensive drugs on the rise in blood glucose caused by ethyl ether anaesthesia of rats has been studied. The blood glucose was determined by the 2-aminobiphenyl method. In the control group the only short initial maximum in blood glucose, 1.34 ± 0.08 mg/ml, was obtained 30 min. after the beginning of ether anaesthesia. Adrenaline injection caused a continuous rise in the blood glucose to 1.72 ± 0.06 mg/ml and showed that the glycogen reserves were present.

In the reserpine group the basal blood glucose content was increased to 1.25 ± 0.05 mg/ml and rose continuously to 1.58 ± 0.10 without the steep initial rise that could be seen in the control group. Adrenaline injection increased the blood glucose content of reserpinised rats from 1.35 ± 0.06 mg/ml to 1.88 ± 0.10 mg/ml.

In the adrenalectomized animals ether anaesthesia did not increase the blood glucose content.

The other antihypertensive drugs, guanethidine, bretylium, and alpha methyldopa, produced a normal or low basal blood glucose content (0.94 ± 0.05, 0.99 ± 0.05 and 0.87 ± 0.02 mg/ml, respectively) and its continuous rise in ether anaesthesia to 1.23 ± 0.09, 1.36 ± 0.10, 1.20 ± 0.08 mg/ml, respectively. In normal rats ether anaesthesia caused only a short initial increase. These antihypertensive drugs, especially α methyldopa, in contradistinction to reserpine, did not increase the basal blood glucose content.

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The Effect of Prolonged Treatment with Noradrenaline in Oil, along with Sodium Chloride and Deoxycortone, on the Blood Pressure of Rats

By

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In studying of aetiology of experimental hypertension, relatively little attention has been paid to the part played by the injections of mediators of sympathetic nervous system in its production, e. g. "prolonged" adrenaline and noradrenaline preparations in oil Adrenaline in water solution and in oil increases slowly the blood pressure during prolonged (ca. 3 months) treatment of rats (HEIM 1952) and during prolonged treatment of dogs (ENGER 1940) An accumulation of hyaline in the slings of the kidney glomeruli and the walls of the small arteries of dogs is seen when the treatment is prolonged to two years (ENGER 1940) But a continuous noradrenaline infusion for several days does not keep the the blood pressure unequivocally increased in rabbits (BLACKET *et al* 1950). Injections of adrenaline in oil or along with sodium chloride treatment in drinking water in our own earlier studies increased the blood pressure of rats over 3 months, and adrenaline along with sodium chloride did so more than the sodium chloride treatment alone Adrenaline along with sodium chloride and desoxycortone or only with desoxycortone increased blood pressure more than sodium chloride or desoxycortone alone (AHO *et al* 1961 AHO *et al* 1962).

The excretion of noradrenaline in the urine lies within normal limits in most patients with essential hypertension (V EULER *et al* 1954 V EULER 1956) in severe hypertension, owing to weakened renal function, it may even be decreased (MÜLLER *et al* 1957) However it is known that sympathetic tone plays a part in the regulation of normal blood pressure as well as in the neurogenic hypertension of phaeochromocytoma. The increased peripheral sympathetic tone of blood vessels t fore seems to be the most probable cause of essential hy
tr

Capillary ischaemia appears after noradrenaline infusion as well as during paroxysmal increase of blood pressure in phaeochromocytoma (GREISMAN 1956 LEE 1956) The effect of noradrenaline upon the circulation resembles the changes in the circulation caused by essential hypertension (PEKKARINEN & HELVE 1952 V EULER 1956), and it could be thought to be an aetiological factor in the mechanism of essential hypertension. The blood pressure can be maintained by both neurogenic and non-neurogenic mechanisms Noradrenaline in the sympathetic nerve ends maintains the peripheral blood vessel tone on the other hand, adrenaline is secreted as an emergency hormone from the adrenal medulla and as a metabolic hormone. In the development of hypertension the peripheral resistance is important, as is also the increased cardiac output in one third of hypertensive patients (BARTORELLI & ZANCHETTI 1961). In hypertension the arterial tone has been observed to be higher than normal (CROUT 1959) In experimental and in human essential hypertension there is a higher than normal reactivity of blood pressure to vasoactive substances and higher than normal vasoconstrictor response in renal and cutaneous blood vessels (BARTORELLI & ZANCHETTI 1961) Desoxycortone strengthens the effect of noradrenaline on the systemic blood pressure in man (RAAB 1953)

There is a certain correlation between the increased blood pressure and emotionality Also in our studies of student examination there was an emotional increase of adrenaline excretion 4–5 times above the normal average (PEKKARINEN *et al* 1961) In the initial stage of essential hypertension psychohygiene, a reduction of conflict situations and psychic strain both in occupational and family life, sufficient rest at night, proper relaxation on Sundays and liberal vacations are known to have a therapeutic effect (MOESCHLIN 1961) Tranquillizers alone are not sufficient to lower the diastolic blood pressure the salt diuretics are more efficient at this. The therapeutic effect of the new saluretics, as well as the increasing frequency of hypertension caused by excessive use of salt, are also well known (CIBA 1961)

The response of the sympathetic nervous system to the modern treatment of essential hypertension with the new drugs also indicates the importance of noradrenaline as an aetiological factor

In the treatment of hypertension, reserpine, guanethidine and α-methyldopa (BRODIE & KUNTZMAN 1960) cause release or reduction in the amount of noradrenaline in sympathetic nerve ends at the same time as they lower the blood pressure. Guanethidine decreases the noradrenaline content of brain and heart (CASS *et al* 1960 BRODIE & KUNTZMAN 1960 COSTA 1961 KRAYER *et al* 1962 KRONEBERG & SCHÜMANN 1962 PFEIFER *et al* 1962)

α-Methyldopa, the dopa decarboxylase inhibitor depletes the catecholamines in brain and heart (PORTER *et al* 1960 HESS *et al* 1960 STONE *et al.* 1961 GOLDBERG *et al* 1960).

Reserpine depletes (PLUMMER 1961) noradrenaline in heart and adrenal glands (CARLSSON & HILLARP 1956 CARLSSON *et al* 1957 PEKKARINEN *et al.* 1958 KRAYER & FUENTES 1958 PAASONEN & KRAYER 1958 WAUD *et al.* 1958) and in brain (BRODIE *et al* 1957) and in sympathetic ganglia (MUSCHOLL & VOGT 1958), as well as in spleen, intestine and liver (PEKKARINEN *et al* 1958) According to HEYMANS & HEYMANS (1957) *sinus caroticus*-denervation increases experimental blood pressure and causes neurogenic hypertension. The new antihypertensive substances can diminish this experimental neurogenic hypertension. Guanethidine diminishes the rise of blood pressure in carotid occlusion. There is also then a tendency for the occurrence of orthostatic hypotension (MAXWELL *et al* 1960). Bretylium tosylate (in 100 mg intravenous doses) causes a blockade of some sympathetically mediated reflexes (BOURA *et al* 1959 BOURA & GREEN 1959 CONWAY 1960 MAXWELL *et al* 1960). Bretylium does not deplete the adrenaline and noradrenaline stores, as guanethidine and reserpine do (COSTA 1961 PEKKARINEN *et al* 1962), but inhibits the spontaneous and reserpine-induced release of noradrenaline (PEKKARINEN *et al* 1962 CALLINGHAM & CASS 1962 HERTTING *et al* 1962) as well as the release of noradrenaline after guanethidine (COSTA 1961 CALLINGHAM & CASS 1962). It acts on the sympathetic nerve terminal by inhibiting the secretion of noradrenaline

In our preliminary test the acute effect of long-acting noradrenaline bitartrate in oil on rats caused a clearer rise in blood pressure than did adrenaline after a single injection of a 1 mg dose in oil (fig. 1 AHO *et al* 1961). In our study we have tried to produce experimental hypertension in intact rats by prolonged treatment with noradrenaline bitartrate in oil only or by prolonged administration of noradrenaline bitartrate and sodium chloride together or noradrenaline bitartrate and deoxycortone We hoped to activate the vasoconstrictive mechanism, the renin mechanism in kidneys or the sodium chloride retaining mechanism of the kidneys by continuous long-acting noradrenaline bitartrate injections, since adrenaline and noradrenaline constrict the renal blood vessels more strongly than other blood vessels.

Material and Methods

We used 44 normal albino rats about 6 months old, their average weight was 200 g In the control group there were 10 rats, in the noradrenaline group 9 rats, in the noradrenaline + sodium chloride group 15 rats and in the noradrenaline + sodium chloride + deoxycortone group 9 rats.

The oil solution of L-noradrenaline bitartrate (levophed ® as base 1 200 or 0.5 g/100 ml, Winthrop products Ltd) contained *acid. ascorb.* 0.015 *cera alba* 0.02, *adeps lan.* 0.04 and *oleum arachidis ad* 1.0 ml) The noradrenaline bitartrate dose, 1 mg/day was injected subcutaneously into the mid-line of the back. The control group received the oil solution with the additional substances but without noradrenaline. The deoxycortone used was deoxycorticosterone oenanthate (primocort depot ®, Schering, Leiras) 25 mg deoxycortone was injected intramuscularly every 10th day Sodium chloride was given as 2 / solution as drinking water

The blood pressure was measured by a photoelectric tensometer (Metro Industries) without anaesthesia (KERSTEN *et al* 1957), the animals being tranquillized for 5–10 minutes in animal holders. The measurement was repeated three times.

As food ordinary laboratory food for rats was given. Most of the animals endured the test fairly well. However 7 of them died during the test, and some became infected at the site of injection in the back. The noradrenaline injections caused fewer infections than the adrenaline in previous corresponding tests (AHO *et al.* 1961 & 1962).

Results

The acute test (10 rats, fig. 1) Noradrenaline bitartrate in oil as a single injection of 1 mg s.c. increased the blood pressure in 2 hours on the average from 110 ± 2.5 mm Hg to 146 ± 3.3 mm Hg. The blood pressure had returned normal in about 6 hours

The control group (10 rats, fig. 2) The initial blood pressure was 111 ± 2.6 mm Hg. After one month s injections of solvent (the oil preparation without noradrenaline) the average pressure was 114 ± 2.8 mm Hg. After two months it was 117 ± 2.2 mm Hg and after 3 months

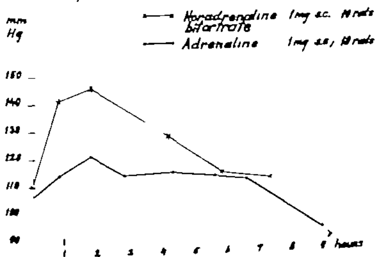


Fig. 1 The effect of single injection of noradrenaline bitartrate in oil (1 mg subcutaneously) on the blood pressure of rats, compared with the effect of adrenaline in oil.

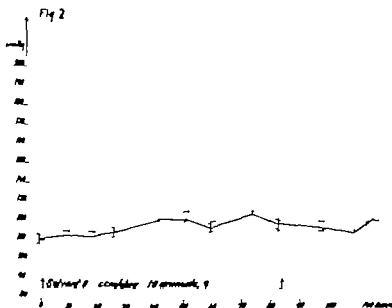


Fig. 2. The effect of prolonged treatment with solvent only on the blood pressure of rats. Individual values and means.

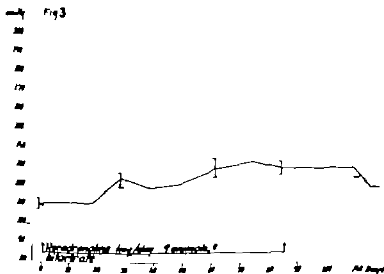


Fig. 3. The effect of prolonged treatment with noradrenaline bitartrate in oil (s.c. 1 mg each day) on the blood pressure of rats.

118 ± 1 7 mm Hg. The maximum blood pressure, 123 ± 3 mm Hg, occurred on the 75th day The maximal rise was thus only 12 mm Hg. During the 2nd and 3rd month of injections the blood pressure was only % of the measurements ≥ 130 mm Hg in 36 / it was ≤ 115 mm Hg.

The noradrenaline bitartrate group (1 mg daily to 9 rats, fig. 3). The

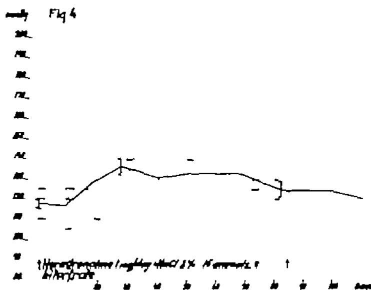


Fig. 4 The effect of combined and prolonged treatment with noradrenaline bitartrate in oil (1 mg. s.c. each day) and 2 % NaCl in drinking water

initial average blood pressure was 111 ± 2.8 mm Hg. After one month's treatment it was 125 ± 3.0 mm Hg. Then the blood pressure decreased somewhat, but it rose again to reach 129 ± 5.1 mm Hg on the 60th day. The increase in blood pressure was significant compared with that of the control group (P = 0.05). After 90 days treatment the blood pressure was 130 ± 4.0 mm Hg and the difference highly significant ($P < 0.01$). The maximal rise in the blood pressure was 22 mm Hg and the maximum blood pressure 133 ± 2.6 mm Hg on the 75th day. After the 2nd and 3rd month of treatment the blood pressure was ≥ 130 mm Hg in 36 % of the measurements. There was no single rise in blood pressure above 150 mm Hg. In 24 % of the measurements the blood pressure was ≥ 115 mm Hg. After the treatment the blood pressure remained increased for 12 days and then decreased slowly.

The noradrenaline bitartrate + sodium chloride group (15 rats, fig. 4). The initial blood pressure was 118 ± 1.9 mm Hg. One month later the blood pressure was 138 ± 3.4 mm Hg and remained at that level until the 68th day when it began to decrease. On the 90th day the average blood pressure was 125 ± 3.8 mm Hg. On the 2nd and 3rd month of injections the blood pressure was ≥ 130 mm Hg in 41 % of the measurements, in 9 % it was ≥ 150 mm Hg, in 4 measurements 160 mm Hg and in one 170 mm Hg. It was ≥ 115 mm Hg in 12 % of the measurements.

The noradrenaline bitartrate + deoxycortone + sodium chloride group (9 rats, fig. 5). The initial blood pressure was 111 ± 2.4 mm Hg. After one

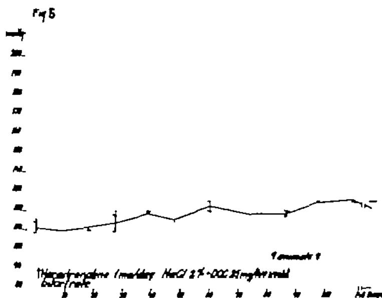


Fig. 5. The effect of combined and prolonged treatment with noradrenaline bitartrate in oil (1 mg s.c. each day) and 2 % NaCl in drinking water and DOC oenanthate (25 mg s.c. every second week) on the blood pressure of rats.

month the blood pressure was only 114 ± 4 4 mm Hg, after two months 122 ± 2.9 mm Hg and after three months 120 ± 1 7 Only in 9 / of the measurements during the 2nd and 3rd months the blood pressure was ≥130 mm Hg, and the highest blood pressure measured was 135 mm Hg.

Discussion

In the aetiology of hypertension there is obviously a relatively long so-called "preparative" phase, before the hypertension begins to develop. Indications are the slowly developing and long hypertensive phase and the higher frequency of hypertension at increasing age. A psychic trauma also may activate hypertension. The blood pressure is maintained by both neurogenic and non-neurogenic mechanisms. Usually the cause of hypertension is multifactorial (SMIRK, personal communication, 1961)

The activity of the sympathetic nervous system, and in that connection noradrenaline, is important in the maintenance of the normal peripheral blood vessel tone. This is indicated among other things by the fact that the ganglion-blocking drugs lower the blood pressure. The experimental studies have given relatively little direct evidence of the importance of noradrenaline in the aetiology of essential hypertension. It has, for instance, not been ascertained whether or not the excretion of line in essential hypertension is higher than normal (v EULER 1956)

some hypertensive patients have a higher than normal noradrenaline excretion (v EULER 1956) Only in neurogenic hypertension, as for instance in phaeochromocytoma, is the secretion of noradrenaline often an aetiological factor (PEKKARINEN & PITKÄNEN 1955 (PITKÄNEN 1957) v EULER 1956 MANGER *et al* 1959 GOLDENBERG *et al* 1954) When the phaeochromocytoma has been removed, the neurogenic hypertension may sometimes continue (RAAB 1953) The excretion of noradrenaline in the urine is lower than normal in severe hypertension (MÖLLER, BUUS & BJERRING 1957) The conditions in our studies with noradrenaline in oil can be compared to those of phaeochromocytoma. The effect of the endogenous noradrenaline coming directly from the nerve ends upon the receptors is difficult to imitate Later we have been able to produce prolonged hypertension in rats after 7 months treatment with adrenaline and noradrenaline adsorbed in glycerol percutaneously (KONU LAAJOKI & PEKKARINEN, unpublished)

In our test the acute blood pressure increase caused by noradrenaline in oil solution (fig. 1) was stronger than that of an equal adrenaline dose. The reaction is, however small compared with the dose of noradrenaline. The increase in blood pressure only lasted for 5–6 hours after the single injection of noradrenaline in oil. It would be an advantage if a noradrenaline preparation of still longer effect could be developed. On the other hand, noradrenaline injections in oil along with sodium chloride treatment in drinking water or with deoxycortone injections were not as effective in increasing the blood pressure of certain animals as adrenaline (AHO *et al.* 1961 & 1962) The rat is somewhat insensitive to adrenaline (GOLDZIEHER 1946)

Adrenocortical hormones, e. g. hydrocortisone and aldosterone have a permissive and modifying role in the aetiology and maintenance of essential hypertension The overproduction of these substances does not generally occur in essential hypertension (GROSS 1962a & b).

An important point about the mode of action of noradrenaline is the duration of renal vasoconstriction, when we consider the possible production of experimental hypertension. Renal vasoconstriction is a very sensitive indication of adrenaline and noradrenaline. As ¼ of the minute volume of the heart passes through the renal circulation, we can understand the importance of renal vasoconstriction in the aetiology of hypertension and in the retention of sodium chloride. Individual sensitivity of the renal circulation may vary considerably It could explain the individual blood pressure rises in some of our test-animals.

Administration of noradrenaline in oil over three months caused a small rise in blood pressure to a level significantly higher than that of the control group although not as much as did prolonged treatment with

adrenaline in oil in our earlier experiments (AHO *et al* 1961). Neither had sodium chloride or deoxycortone the same potentiating effect upon the increase of blood pressure caused by noradrenaline as upon that caused by adrenaline. *An explanation to this can perhaps be found in the diuretic* effect caused by small doses of noradrenaline and the fact that adrenaline is a stronger vasoconstrictor for the kidneys than noradrenaline. The small blood pressure increase produced by noradrenaline and sodium chloride in chronic tests is remarkable, if we take into account the fact that the new antihypertensive drugs e g. guanethidine, bretylium and α-methyldopa as well as reserpine decrease the blood pressure by influencing upon the noradrenaline releasing mechanism from the nerve ends on the noradrenaline content or the decrease of the content of noradrenaline in tissues (BRODIE & KUNTZMAN 1960 CONWAY 1960 RICHARDSON & WYSO 1960 SJOERDSMA 1960) a decrease in blood pressure that is still enhanced by the saluretics or the avoidance of extra salt (FURNESS 1960)

Depending on the degree of vasoconstriction and of the blood pressure, noradrenaline can be either diuretic or antidiuretic. An increased blood pressure can promote diuresis. Too great an increase in blood pressure and a simultaneous vasoconstriction can, however decrease diuresis.

In *our earlier tests, on the other hand, adrenaline alone or together with* sodium chloride and deoxycortone clearly increased the blood pressure (AHO *et al* 1961 & 1962) There is only a difference in degree between hypertensive and normotensive individuals.

Summary

The combined effect upon the blood pressure of subcutaneous injections of noradrenaline bitartrate in oil (1 mg daily) and of 2 % sodium chloride in drinking water as well as of noradrenaline bitartrate alone and of the combination of noradrenaline bitartrate, deoxycortone and sodium chloride was studied during 3 months in 44 normal rats. The control group was treated with oil solvent alone. The blood pressure was measured without anaesthesia and without bleeding by a photoelectric tensometer In the noradrenaline bitartrate group the mean control blood pressure (111 $\pm$ 2.8 mm Hg) increased in 75 days 22 mm Hg (to 133 $\pm$ 2.6 mm Hg) The increase in the mean blood pressure was small after the second and third month of treatment there were values $\geq$130 mm Hg in 36 % of the measurements. There was no single blood pressure rise over 150 mm Hg. In the noradrenaline bitartrate and sodium chloride group the initial blood (118 $\pm$ 1 9 mm Hg) increased in one month to 138 $\pm$ 3 4 remained nearly at that level during the second month and

125 ± 3 8 mm Hg during the third month. During the second and third month the blood pressure was ≥ 150 mm Hg in only 9 / of measurements. In the control group after injections of oil solvent alone the blood pressure (111 ± 2.6 mm Hg) increased in 2 and 3 months to 118 ± 1 7 mm Hg. The combination of *noradrenaline* bitartrate, deoxycortone and sodium chloride increased the blood pressure (111 ± 2.4 mm Hg) only slightly in two months to 122 ± 2.9 mm Hg. Only in 9 / of the measurements during the second and third months were the blood pressures ≥ 130 mm Hg.

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Prolonged Decrease in Vanilmandelic (3–Methoxy–4–Hydroxy mandelic) Acid Excretion by Man during and after Nialamide Treatment

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The monoamine oxidase (MAO) inhibitors increase the excretion of both exogenic (CORNE & GRAHAM 1957 CRAWFORD & LAW 1958) and endogenic (PEKKARINEN *et al* 1960) adrenaline and noradrenaline in some test animals, though not always (CRAWFORD & LAW 1958), and not in man (CARLSSON *et al* 1959 BRUNJES *et al* 1963).

In man vanilmandelic acid (VMA) forms most of the total adrenaline and noradrenaline metabolites excreted (ARMSTRONG *et al.* 1957 AXELROD 1960 LABROSSE *et al* 1961 ZIEGLER 1960 GOODALL & ROSE 1963) The MAO-inhibitors decrease its excretion after an adrenaline infusion in test animals (AXELROD 1959 KIRSHNER 1960) and in man (GOODALL *et al* 1958 RESNICK *et al* 1958 GOODALL 1959) The excretion of vanilmandelic acid decreases during MAO-inhibitor treatment of short duration (VON STUDNITZ 1959 ZIEGLER 1960 ISHIDA 1962), and as a result of 0-methylation the excretion of metanephrine and normetanephrine increases by the test animals (KRAUPP *et al* 1962) and by man (CROUT *et al* 1961 ISHIDA 1962 BRUNJES *et al.* 1963)

In our work the effect of prolonged treatment with the MAO-inhibitor nialamide on the excretion of vanilmandelic acid in man was studied, and the duration of the effect after treatment was followed.

Material and Methods

The material consisted of 50 hospitalized chronic anergic male schizophrenic patients (the average age 45 years). Most of them had been in hospital for several years. There has not been ascertained any renal or liver disease.

To 20 of the patients were administered orally 300 mg of nialamide (Niamid ®, Pfizer), divided into three doses daily for 5 weeks. For at least 2 weeks before

beginning of and during the nialamide treatment the patients had no other medication. The control group consisted of 20 other patients (10 patients had no antidepressive treatment and 10 other patients had been given clopenthixol (Sordinol ® Lundbeck & Co) from 30 to 75 mg a day). In 10 other control patients the VMA-excretion was studied 178 days after the nialamide treatment.

The daily urine was always collected for two consecutive days, immediately before the beginning, 3 times during and 6 times after the nialamide treatment. The last collection was performed 6 months after the treatment (see table 1). Vanilmandelic acid (VMA, 3-methoxy-4-hydroxymandelic acid) was determined by a micromodification (PEKKARINEN & HAKULINEN unpublished) of PISANO *et al* (1962) method on 0.8 ml urine for VMA and its blank. Two volumes of periodate and bisulphite and prolonged intensive extraction times were used to improve the quantitative recovery. The standard error of the mean for added VMA (8 μg) is ± 2.4% as an average of 13 individual series (PEKKARINEN *et al.* 1963).

The recovery of VMA, added as the method standard to the urine was 76.8 %, 78.5 % and 82.6 % for 4,8 and 16 μg of VMA, respectively as compared to the colour reaction in KOH for the equivalent amount of vanillin. Standard deviation for the added method standard in present series is ± 5.4 %, ± 2.8 % and ± 1.6 %, respectively (4,8 and 16 μg VMA).

Results

1 *The control excretion of VMA* (tables 1 and 2).

The nialamide group The average diurnal excretion of VMA in the male anergic schizophrenics before nialamide treatment was 4.4 and 4.6 mg for two consecutive days.

The group without nialamide treatment (table 3). In the control group the excretion of VMA was 4.1 and 4.1 mg in the patients receiving clopenthixol treatment and correspondingly 4.3 and 3.6 mg a day in those patients receiving no medical treatment. In the later control group of 10 patients it was 4.9 and 4.2 mg a day. This was the control groups used to determine the level of excretion in the nialamide group 6 months after treatment.

2. *The VMA excretion during nialamide treatment* (tables 1 and 2)

Nialamide treatment with 300 mg a day for 8 days decreased already significantly ($P < 0.001$) the VMA excretion to 2.7 and 3.0 mg per day (decreases of 39 and 35 %). During the treatment the VMA excretion decreased continually. After 15 days it was 2.1 and 2.1 mg a day (decreases of 52 and 54 %) and after 35 days treatment 1.2 and 1.3 mg a day (decreases of 73 and 72 %). The VMA excretion generally decreased uniformly during treatment except in 3 patients (Nos. 3, 7 and 16) whose excretion had decreased by 8 days to the level at which it remained during the whole treatment.

Table 1

Effect of monoamine oxidase inhibitor nialamid (300 mg/day) on the excretion of vanilmandelic acid (mg/24 h) in chronic anergic schizophrenic patients. Mean ± S.E.M

	Pretreatment Control	Time of treatment (days)			Time of follow-up period (days)						Control Clopenthixol		Control Late
Days		8	15	35	17	48	63	93	123	177	With	Without	
Day I	4.4	2.7	2.1	1.2	2.6	3.0	2.4	2.9	3.3	2.8	4.1	4.3	4.9
	±0.33	±0.28	±0.28	±0.24	±0.27	±0.44	±0.25	±0.35	±0.53	±0.16	±0.40	±0.60	±0.60
n	(17)	(18)	(16)	(14)	(15)	(14)	(13)	(12)	(13)	(13)	(10)	(10)	(10)
Day II	4.6	3.0	2.1	1.3	2.6	2.7	3.4	3.1	3.3	3.5	4.1	3.6	4.2
	±0.50	±0.35	±0.28	±0.18	±0.26	±0.37	±0.46	±0.36	±0.29	±0.50	±0.25	±0.62	±0.42
	(18)	(17)	(18)	(14)	(14)	(14)	(11)	(12)	(13)	(11)	(10)	(9)	(10)
Mean of I and II day	4.3	2.8	2.0	1.2	2.7	2.8	2.8	3.0	3.3	3.1	4.2	4.1	4.6
	0.32	±0.28	±0.26	±0.17	±0.22	±0.38	±0.24	±0.30	±0.39	±0.26	±0.27	±0.57	±0.48
P		0.001[1])			0.001[2])				0.05[1])	0.01[3])			

) P values, when the mean of two days excretion is compared with the pretreatment control.
[2]) P value, when the mean of two days excretion is compared with the mean of 35 days treatment.
[3]) P value, when the mean of two days excretion is compared with both the pretreatment and the late control group.

Table 2

The effect of monoamine oxidase inhibitor (nialamide) on the excreti n of vanilmandelic cid (mg/24 h) in chronic anergic schizophrenic patients. *Individual values.*

Number of patient	Pretreatment Control		Time of treatment (days)						Time of follow- p period (days)											
			8		15		35		17		48		63		93		123		177	
	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II
1	3.0	1.0	1.2	1.0		–	–	–	–	–	–	–		–	–	–	–	–	–	–
2	4.0	10.6	3.7	6.0	3.7	3.0	2.4	1.9	4.1	2.8	3.5	3.2	2.0	2.9	2.5	3.1	2.5	2.7	2.1	2.1
3	2.3	2.5	1.4	1.3	1.3	1.2	–	–	1.8	4.0	0.9	1.1	2.2	3.9	–	–	1.7	4.0	3.0	2.4
4		4.2	3.7	3.0	1.4	2.5	0.5	0.4	3.2	3.4	4.2	3.9	2.0	3.4	2.0	4.8	1.7	3.5	2.9	4.6
5	3.5	3.5		–	0.6	1.6	0.4	0.2	1.6	0.4	0.9	1.0	0.9	1.8	1.1	1.1	2.9	2.4	2.4	0.4
6	2.4	8.5	2.9	4.4	1.4	2.5	0.6	1.2	1.0	2.9	4.9	3.9	1.5	2.4	3.5	3.9	3.0	4.3	2.2	5.0
7	4.3	4.5	1.6	1.7	2.1	1.1	0.3	1.5	1.9	2.6	2.4	3.8	3.2	4.4	3.0	4.0	5.0	3.3	2.5	3.8
8	5.0	3.5	4.2	2.2	2.5	2.7	0.8	1.9	3.9	3.3	–	–	–	–	–	–	–	–	–	–
9	4.8	4.6	2.5	2.8	2.5	2.2	1.7	1.8	3.1	3.2	5.9	3.3	3.6	3.5	4.2	1.6	5.8	5.0	4.3	4.4
10		–	1.0	–	–	0.6	0.5	0.3	1.0	1.6	1.4	1.9	1.9	–	1.4	3.5	0.5	0.7	2.6	–
11	3.1	4.3	4.4	2.6	3.4	3.2	1.7	2.2	2.5	1.4	3.9	5.9	1.5	7.4	4.0	4.2	6.7	3.7	3.0	5.8
12	4.5	6.[illegible]	3.8	5.1	3.7	5.4	3.6	1.6	4.3	3.1	4.2	2.7	4.1	3.5	3.0	1.9	5.4	4.3	3.1	4.5
13	5.1	2.3	1.6	2.6	1.0	2.2	1.3	1.3	2.3	3.3	2.7	1.7	2.8	2.5	5.3	4.8	1.3	3.1	2.8	3.8
14	5.2	3.6	2.2	5.0	3.5	1.6	1.2	2.0	3.5	3.2	1.7	1.8	3.0	2.0	2.2	3.1	2.6	3.4	2.4	1.6
15	–	–	2.5	3.0	0.6	0.6	1.1	0.9	3.1	–	0.9	1.4	2.3	–	2.3	1.0	3.7	3.0	3.0	–
16	2.8	3.5	1.0	1.3	0.7	0.7	1.1	0.7	2.4	1.6	3.9	2.0	–	–	–	–	–	–	–	–
17	5.8	5.4	3.7	2.6	1.4	3.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	6.9	5.8	4.3	3.9	3.7	2.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	5.8	3.9	4.4	1.8	–	1.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20	6.0	4.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

D I = 1st day D II = 2nd day

Table 3

Excretion of vanilmandelic acid (mg/24 h) I control patients. Individual values.

Number of patient	Clopenthixol				Late control	
	With		Without			
	D I	D II	D I	D II	D I	D II
1	3.4	2.9	3.3	3.8	6.5	4 8
2	6.6	4.5	0.6	1 3	5.8	3.2
3	4.3	4.0	3 8	4.5	7.8	5.1
4	2.8	3.9	6.0	5 1	5.9	6.5
5	3.2	3.0	2.7	2.9	3.5	3.9
6	4.1	4.9	5.6	2.3	3 5	3 6
7	3.5	5.5	6.9	7.5	1 9	1.9
8	3.4	3 9	4 1	2.4	4 9	3 4
9	6.1	4 6	4 0	2.6	2.7	3.9
10	3 7	4.0	6.1	–	6.4	5 7

D I = 1st day D II = 2nd day

3 *The VMA excretion after nialamide treatment* (tables 1 and 2)

After treatment the excretion of VMA rose gradually This rise was greater during the first 17 days (the excretion was then 2 6 and 2.6 mg a day). The excretion 123 days after the treatment was 3 3 and 3 3 mg a day and 177 days after the treatment still 2.8 and 3 5 mg a day Compared to the pretreatment control excretion the 123 days mean value was still lowered ($P < 0.05$). The excretion 177 days after the treatment was significantly decreased ($P < 0.01$) compared to the simultaneous control without nialamide treatment (table 3) and also compared to the pretreatment value ($P < 0.01$). In two patients (Nos. 5 and 10) the increase in excretion took place considerably more slowly than in the others.

4 *The psychic effect of the nialamide treatment*

In 10 of the nialamide treated patients (Nos. 1–10) a clear increase in psychic activity was observed after a 2–3 weeks treatment. This, however led to restlessness and even slight agitation in all these patients. Of these one (No 1) became so aggressive after 4 weeks treatment that the treatment could not be continued. Moreover the hallucinations were activated in two of the patients (Nos. 7 and 9) The stimulating effect of the treatment could as a rule still be observed about two weeks after nialamide administration had ended In the above-mentioned patient (No 1) who became aggressive these symptoms did not disappear until 4 weeks later

In the patients in whom the nialamide treatment caused a clear

tion, all but one (No 9) had during the whole time of

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Table 2

The effect of monoamine oxidase inhibitor (nialamide) on the excretion f vanillmandelic acid (mg/24 h) in chronic anergic schizophrenic patients. Individual values.

Number of patient	Pretreatment Control		Time of treatment (days)						Time of follow-up period (days)											
			8		15		35		17		48		63		93		123		177	
	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II	D I	D II
1	3.0	1.0	1.2	1.0	–		–	–		–	–		–	–	–	–	–	–	–	–
2	4.0	10.6	3.7	6.0	3.7	3.0	2.4	1.9	4.1	2.8	3.5	3.2	2.0	2.9	2.5	3.1	2.5	2.7	2.1	2.1
3	2.3	2.5	1.4	1.3	1.3	1.2	–	–	1.8	4.0	0.9	1.1	2.2	3.9	–	–	1.7	4.0	3.0	2.4
4	–	4.2	3.7	3.0	1.4	2.5	0.5	0.4	3.2	3.4	4.2	3.9	2.0	3.4	2.0	4.8	1.7	3.5	2.9	4.6
5	3.5	3.5		–	0.6	1.6	0.4	0.2	1.6	0.4	0.9	1.0	0.9	1.8	1.1	1.1	2.9	2.4	2.4	0.4
6	2.4	8.5	2.9	4.4	1.4	2.5	0.6	1.2	1.0	2.9	4.9	3.9	1.5	2.4	3.5	3.9	3.0	4.3	2.2	5.0
7	4.3	4.5	1.6	1.7	2.1	1.1	0.3	1.5	1.9	2.6	2.4	3.8	3.2	4.4	3.0	4.0	5.0	3.3	2.5	3.8
8	5.0	3.5	4.2	2.2	2.3	2.7	0.8	1.9	3.9	3.3		–	–	–	–	–	–	–	–	–
9	4.8	4.6	2.5	2.8	2.5	2.2	1.7	1.8	3.1	3.2	5.9	3.3	3.6	3.5	4.2	1.6	5.8	5.0	4.3	4.4
10		–	1.0	–		0.6	0.5	0.3	1.0	1.6	1.4	1.9	1.9	–	1.4	3.5	0.5	0.7	2.6	–
11	3.1	4.3	4.4	2.6	3.4	3.2	1.7	2.2	2.5	1.4	3.9	5.9	1.5	7.4	4.0	4.2	6.7	3.7	3.0	5.8
12	4.5	6.2	3.8	5.1	3.7	5.4	3.6	1.6	4.3	5.1	4.2	2.7	4.1	3.5	3.0	1.9	5.4	4.3	3.1	4.5
13	5.1	2.3	1.6	2.6	1.0	2.2	1.3	1.3	2.3	3.3	2.7	1.7	2.8	2.5	5.3	4.8	1.3	3.1	2.8	3.8
14	5.2	3.6	2.2	3.0	3.5	1.6	1.2	2.0	3.5	3.2	1.7	1.8	3.0	2.0	2.2	3.1	2.6	3.4	2.4	1.6
15	–	–	2.5	3.0	0.6	0.6	1.1	0.9	3.1	–	0.9	1.4	2.3	–	2.3	1.0	3.7	3.0	3.0	–
16	2.8	3.5	1.0	1.3	0.7	0.7	1.1	0.7	2.4	1.6	3.9	2.0	–		–	–		–	–	–
17	5.8	5.4	3.7	2.6	1.4	3.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	6.9	5.8	4.3	3.9	3.7	2.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	5.8	3.9	2.4	1.8	–	1.1	–	–		–	–	–	–	–	–	–	–	–	–	–
20	6.0	4.9	–	–	–	–	–	–	–		–	–		–	–	–	–	–	–	–

D I = 1st day D II = 2nd day

uptake of the circulating catecholamines into the liver cells (INNES 1963). The inhibition of adrenaline and noradrenaline release from adrenergic nerves, as indicated by iproniazid treatment of hypertensive patients, might also partly explain our findings on the decrease in excretion of their main metabolite, VMA. However nialamide does not as clearly decrease blood pressure during treatment as iproniazid. The determination of the excretion of VMA during treatment with MAO inhibitors always provides further possibilities for evaluating the magnitude of the metabolic inhibition of adrenaline and noradrenaline.

Summary

With chronic anergic schizophrenic patients the effect of a 5 weeks MAO inhibitor (nialamide) treatment upon the excretion of vanilmandelic acid (VMA) and the duration of that effect after the treatment were studied.

Before the treatment with nialamide the average diurnal excretion was for two consecutive days 4.4 and 4.6 mg. An 8 days treatment (300 mg/day) decreased the excretion to 2.7 and 3.0 mg/day ($P < 0.001$) for two consecutive days (a decrease of 39 and 35 %). The decrease continued during the whole period of treatment to 1.2 and 1.3 mg/day after 35 days treatment. After the nialamide treatment the VMA excretion increased gradually in 17 days to 2.6 and 2.6 mg and after 123 days to 3.3 and 3.3 mg/day. Then the difference also is slightly significant ($P < 0.05$). At 177 days after the nialamide treatment the excretion was still 2.8 and 3.5 mg/day or slightly decreased compared to the pretreatment value ($P < 0.01$) or to the simultaneous late control group ($P < 0.01$).

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Biochemistry and Toxicology of Amikapron ®, The Antifibrinolytically Active Isomer of AMCHA. (A Comparative Study with ε-Aminocaproic Acid)

By

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OKAMOTO *et al* (1962a, b c), after their introduction of ε-aminocaproic acid (EACA) as an antifibrinolytic drug, continued their search for more potent agents and selected *p*-aminomethylcyclohexane carboxylic acid (AMCHA) for further evaluation. The chemical formulae are given in fig. 1



EACA



AMCHA

Chemical formulae of ε-aminocaproic acid (EACA) and p- aminomethyl cyclohexane carboxylic acid (AMCHA)

Figure 1.

Having studied for a number of years aminocaproic acid in a variety of test systems, we became interested in Okamoto s recent contributions and initiated investigations on AMCHA. Analysing AMCHA chemically we found that the synthetic material was not uniform, but comprised two isomers. The synthetic pathways described in the chemical literature for AMCHA result in a mixture containing from about 10/ to about 25/ of an isomer that we found to possess all the antifibrinolytic activity of the mixture. The two isomers have been separated, characterized and studied

in a series of test systems. The compounds were studied in the form of amikapron ® (active AMCHA isomer) and epsikapron ® (aminocaproic acid) preparations.

OKAMOTO *et al* (1964) have confirmed that their first studies were made with a similar AMCHA mixture of isomers.

Methods and Results

A. *Antifibrinolytic activity in vitro*

The procedures used are described by CHRISTENSEN (1949) and WASSERMAN *et al* (1953) They involve measuring the amount of active AMCHA isomer necessary for producing 50 % inhibition of streptokinase-induced fibrinolysis *in vitro* The system consists of fibrinogen, plasminogen and thrombin, to which is added 6 units of streptokinase, which produces lysis. The 50 % inhibition determined corresponds to a lytic activity of 3 units of streptokinase Results are presented in table 1

B. *Serum levels in and urinary excretion by rabbits*

Serum samples are deproteinized by pressure dialysis, and the filtrates are freeze-dried The resulting residues are re-dissolved in small volume of distilled water the amino acids are separated and identified by the ninhydrin reaction, with elution and spectrophotometric assay

Results on serum levels from groups of three rabbits injected with 100 mg/kg of the various drugs are presented in table 2.

Results of urinary excretion of the drugs and of possible metabolites are given in table 3 Groups of from three to six rabbits injected with 250 mg/kg of the drugs have been studied for 72 hours.

In a parallel series the antifibrinolytic activity of serum from groups of three rabbits has been studied *in vitro* against streptokinase (kabikinase ®)

After a control blood sample, aminocaproic acid and AMCHA at dose

Table 1

Antifibrinolytic activity *in vitro*.
Expressed as 50 % inhibition of 6 units of streptokinase
(6 USK kabikinase ®)

Drug	Relative Activity	True Amount of Drug
Aminocaproic Acid	1	4 µg
AMCHA containing 10 active isomer	2	2 µg
Amikapron ®, Active AMCHA isomer	20	0.2 µg
Inactive AMCHA isomer	<0.1	>30 µg

Table 2

Serum levels in groups of three rabbits after intravenous injection of 100 mg/kg at zero time.

Serum levels, mg/100 ml, at various times after injection. *Range of values within brackets.*

Drug	After 5 min.	60 min.	240 min.
ε-Aminocaproic Acid	19.2 (18.5–20 4)	5.9 (4.3–7 1)	0.4 (0.0–1 1)
AMCHA	23.8 (21 0–27.5)	8.6 (6.8–9.8)	2.2 (1.0–4.5)
Amikapron ®, (active AMCHA isomer)	16.6 (15 8–17.8)	5.5 (5.0–6.2)	1 0 (0.8–1.3)

Table 3

Urinary recovery in groups of three to six rabbits after intravenous injectio of 250 mg/kg at zero time.

Per cent recovery t various times after injection. Range of values within brackets.

Drug	After 24 h	48 h	72 h	Total recovery per cent
ε-Aminocaproic Acid	46 (11–72)	57 (43–79)	Same as at 48 h	57 (43–79)
AMCHA	57 (51–64)	61 (54–69)	Same as t 48 h	61 (54–69)

Parallel excretion of ctive and inactive isomers.
No metabolites detected.

levels of 10, 25 50 and 100 mg/kg were administered intravenously Serum samples were tested for antifibrinolytic activity *in vitro* The time intervals were 4 6, 7.5 18 24 30 and 48 hours after administration of the drugs.

The results are in agreement with those on serum levels and urinary excretion, and they thus confirm the rapid disappearance of the activity from the blood

C. *Absorption studies*

In order to study absorption, AMCHA was given orally at dose levels of 1000 and 1500 mg/kg to rats. Serum levels of the drug were determined between 1 and 17 hours after administration. The results are presented in table 4

D *Toxicology*

The acute toxicities of aminocaproic acid, the synthetically obtained mixture of AMCHA isomers, and the antifibrinolytically active AMCHA

Table 4

Absorption of AMCHA in Sprague-Dawley (Anticimex) rats after oral administration, as judged by serum levels.

Serum levels (γ/ml) t various times after oral administration of 1500 and 1000 mg/kg. Total number of rats 36. Range of values within brackets.

Drug mg/kg	After 1 h	3 h	4 h	5 h	7 h	17 h
AMCHA 1500	27 (14–37)	63 (14–100)	65 (35–96)	32 (20–45)	32 (20–45)	19 (10–35)
AMCHA 1000	–	–	38 (16–46)	–	–	< 10

Table 5

Acute toxicity LD50 (mg/kg), 48 hours after dministration. Number of animals within brackets.

Drug	i.	p. o.
Mouse		
ε-Aminocaproic acid	3000 (90)	12,000 (50)
AMCHA	1600 (70)	> 15,000 (30)
Amikapron ®, (active AMCHA isomer)	1500 (40)	–
Rat		
ε-Aminocaproic acid	3200 (50)	16,500 (20)
AMCHA	1800 (60)	> 15,000 (14)
Amikapron ® (active AMCHA isomer)	1200 (40)	Available preparations limit administration to 3000 mg/kg.

isomer were determined as LD50 values at 48 hours after intravenous or oral administration. Results are given in table 5

In order to establish whether or not accumulation of aminocaproic acid and AMCHA takes place in the animal organism, two studies were carried out on mice and rats, in groups of ten. In one of these approximately one fourth of the calculated LD50 value was given on four consecutive days, and any resulting mortality was recorded. In the second of these the experimentally found LD50 value was given four times.

No mortality due to accumulation of the drugs was recorded. No pathological changes were found[1]) No abnormal values for serum glucose, SGOT SGPT or alkaline phosphatase were obtained

[1]) Liver heart, kidneys and lungs. Courtesy of Dr Gunnar Nyström, Vet. Surgeon, National Bacteriological Laboratory Stockholm, Sweden.

E. Influence of AMCHA and EACA on fertility conception and foetal development in the rat

The Experimental design was a combined two-generation and chronic toxicity study in the rat Mature white rats of the Sprague-Dawley (Anticimex) strain of both sexes and with 10 + 10 animals per dose group were used. Double sets of female rats were used, one of the sets being used in the study of the drug effect on conception and foetal development and the other for a normal chronic toxicity study of three months duration.

The study was a comparative one, with ε-aminocaproic acid as a standard and with a control group receiving water in the same manner as the drugs.

The lowest dose chosen was 500 mg/kg p o daily This approximates to the highest daily dose used clinically for aminocaproic acid. The next higher dose level was 2500 mg/kg p o daily and the highest dose level studied was 5000 mg/kg p o daily The same dosage schedule was employed for both aminocaproic acid and AMCHA.

The AMCHA material used contained about 25 % of the active isomer In acute toxicity studies similar LD50 values were obtained for the naturally occurring isomer mixture and for a preparation containing 95 % of the active AMCHA isomer Thus, with a similar degree of toxicity for aminocaproic acid and AMCHA, the safety index will be considerably higher for AMCHA than for aminocaproic acid Further the same excretion pattern exists for the two compounds. It has, therefore, been considered adequate to use AMCHA instead of the active isomer in this study especially since the same dosage schedules were chosen for AMCHA and aminocaproic acid.

Conduct of study

The compounds were administered in aqueous solution, and the controls were given orally 20 ml water per kg daily Groups of ten animals were used, and two sets of female rats were used at each dose level in order to satisfy the design of the study as a combined two-generation and chronic toxicity test for a longer period

The animals were treated for one month, and one set of the female rats was then allowed to mate with males from a corresponding dosage group. Vaginal smears were taken daily and the males were removed as soon as conception had been confirmed. The drugs were administered also during the mating period, which was extended to ten days in order to cover the oestrous cycle When pregnancy did not occur during this ten day period, the result of the mating has been considered negative.

On the 21st day after a positive vaginal smear cesarean section was

performed, and the young were studied as well as the mother Gross[1])- and histo-pathological[2]) examination were carried out on foetuses and mothers. The foetuses were then cleared, and the skeletons were stained with alizarine red by the method of LORKE (1963) Histo-pathology was performed on the mothers (lung, heart, kidneys, liver)

Further blood and urine samples were taken for clinical chemistry[3]) in the same way as was adopted for the chronic toxicity studies.

The female rats of this two-generation study thus received drug treatment for at least one month and twenty-one days and most of them for two months. The males and the parallel set of females continued to receive the compounds during three full months for the chronic toxicity part of the investigation. These results will be discussed separately under the heading Chronic toxicity"

The results from the two-generation study are presented in tables 6 and 7

F *Chronic toxicity*

As indicated in the description of the two-generation study groups of Sprague-Dawley (Anticimex) rats of both sexes were treated orally for three months with aminocaproic acid and the synthetically obtained mixture of AMCHA isomers. The use of the isomer mixture instead of the antifibrinolytically active AMCHA isomer was justified by the close relationship between the mixture and the pure isomer in their acute toxicities and excretion rates. When planning these studies we disregarded the fact that the active AMCHA isomer is twenty times more potent than aminocaproic acid as an antifibrinolytic agent, and the same dose levels were chosen for both drugs.

The animals were dosed five days weekly for three months at dose levels unrealistically high from a practical point of view but chosen so as to produce detectable changes in the groups.

The results obtained are presented in tables 8 and 9 In table 8 males and females have been treated as one homogenous group, but in table 9 the results have been broken down for the two sexes.

G *Anti-anaphylactic and anti-allergic activity*

Claims have been made that aminocaproic acid possesses anti-anaphylactic properties. Based on the studies of KALLOS & MELANDER (1959)

[1]) Courtesy of Dr Gunnar Nyström, Vet. Surgeon, National Bacteriological Laboratory Stockholm, Sweden

[2]) Courtesy of Dr S. E. Björklund, D.V.M Assoc. Prof., Royal Veterinary College, Stockholm, Sweden.

[3]) Courtesy of Dr B. Åberg, M.D., Assoc. Prof Clin. Lab., Royal Veterinary College, Stockholm, Sweden.

Table 6

Two-generation study Adult female rats.

Ten Sprague Dawley (A ticimex) female mother rats per dose group. Mg/kg/day p. o. for up to two months 500 2500 nd 5000.

Gross pathology It has not been possible to produce lesions and mortality with the medications studied. No gross pathological changes were found that can be referred t the drugs under study

Histo-pathology N pathological tissue damage of the livers, hearts, lungs or kidneys were found in the histo-pathological examination to be connected with the medications administered.

Statistical evaluation of laboratory results[1])

Significant differences between the values for treated and controls were obtained for the following pairs of female rats

Dose mg/kg/day p. o.	ε Aminocaproid acid — Figure for treated group — Higher than controls	ε Aminocaproid acid — Figure for treated group — Lower tha controls	AMCHA — F gure f r treated group — Higher than controls	AMCHA — F gure f r treated group — Lower than controls
500	No significant changes versus controls		No significant changes versus controls	
2500	Alk. phosph. Haemoglobin Red cells Haematocrit Immatures (Indication f dehydration)	Body weight SGPT		Immatures
5000	Haemoglobi Red cells Haematocrit Eosinophils (Indication of dehydration)	Body weight Liver weight Heart weight SGPT Neutrophil	Alk. phosph. Haemoglobin Immatures	

[1]) Laboratory data vailable for evaluatio Weights of body liver heart, lung, kidney SGOT SGPT glucose, alk. phosphatase, serum creatinine, haemoglobin, red cells, haematocrit, thrombocytes, white cells, banded neutrophils, segmented neutrophils, eosinophils, lymphocytes, monocytes, basophils, immatures.

we have developed a reproducible test involving sensitizing mice with horse serum and subsequent challenge with the same foreign protein.

The mice are sensitized by subcutaneous injection of 1 ml of horse serum repeated four times in one week. Beginning on the day after the fourth injection the antifibrinolytically active AMCHA isomer is given orally for two and three weeks, respectively at the dose levels of 50 and 500 mg. Intravenous challenge by 1 ml of horse serum is given one month after the first horse serum injection. The results are presented in table 10.

As a further control on any possible anti-allergic effect of the active AMCHA isomer 100 mg/kg s.c. were given to a group of 4 guinea pigs

Table 7

Two-generation study Foetal development.

Ten Sprague-Dawley (Anticimex) female mother rats per dose group.
Mg/kg/day p. o. for up to two months 500 2500 and 5000.

CONTROLS	Pregnancy in nine of ten females. Two resorptions. Minor developmental disturbances in four cases.
	500 mg/kg/day p. o.
ε-Aminocaproic acid	Pregnancy in six of ten females. No deviatons versus controls.
AMCHA	Pregnancy i nine of ten females. N deviations versus co trols.
	2500 mg/kg day p
ε-Aminocaproic acid	Pregnancy in three of ten females. One foetus displ ying double set-up of hind legs.
AMCHA	Pregnancy in six of ten females. No deviations versus controls.
	5000 mg/kg/day p. o
Aminocaproic acid	No pregnancy!
AMCHA	Pregnancy i five f ten females. No deviation versus controls.

exposed to a histamine aerosol The time for development of bronchospasm was the same in the treated group as in the control group of 4 guinea pigs.

Discussion

The observation that the antifibrinolytic activity of AMCHA is exerted by one of its stereoisomers opens the route to a drug with a potency considerably above that of aminocaproic acid

DUBBER, MCNICOL & DOUGLAS (1964a, b) found the active AMCHA isomer to be about one hundred times as potent as aminocaproic acid *in vitro* in a urokinase activated system. OKAMOTO *et al* (1964), in a system similar to the one used by us, found the active AMCHA isomer to be 26 times more potent than aminocaproic acid, a figure of the same order as we have found

In rabbits we found serum levels and excretion rate to be about the same for aminocaproic acid and AMCHA. In studies in which an isomer mixture was used, a parallel excretion of the active and the inactive isomers was recorded. We were unable to find any metabolites detectable by ninhydrin.

As judged by the absorption studies in rats AMCHA is rapidly sorbed after oral administration.

Table 8

Chronic toxicity – rat.

Ten Sprague-Dawley (Anticimex) rats of both sexes per dose group. Mg/kg/day p.o. for three m ths 500 2500 and 5000.
Gross pathology It has not been possible to produce lesions and mortality with the medications studied. N gross pathological changes were found to be referred to the drugs der study

Histo-pathology No pathological tissue damages of the livers, hearts, lungs, or kidneys have been found in the histo-pathological examination which can be connected with the medications administered.

Clinical chemistry

Statistical evaluation of laboratory results[1])
Significant differences between the values for treated and controls were obtained for various pairs.

Dose mg/kg/day p. o.	ε-Aminocaproic acid: Figure f treated group				AMCHA: Figure for treated group			
	p-value	Higher than controls	p-value	Lower than controls	p-value	Higher than controls	p-value	Lower than controls
500	0.0[illegible]–0.01	Heart weight	0.05–0.02	Glucose	0.05–0.02	SGPT	0.01–0.001	Thrombocytes
	0.01–0.001	Red cells	0.05–0.02	Serum creatinine	0.001	White cells	0.05–0.02	Eosinophils
	0.05–0.02	White cells			0.05–0.02	Haematocrit		
					0.05	Neutrophil. segm.		
2500	0.05–0.02	Liver weight	0.02–0.01	SGOT	0.01–0.001	SGPT	0.02–0.01	Haemoglobin
	0.05–0.02	Heart weight	0.001	Glucose	0.001	Alk. phosph.		
	0.001	Alk. phosph.	0.05–0.02	Serum creatinine	0.05–0.02	Haematocrit		
	0.01–0.001	Neutrophil. segm.	0.05–0.02	Thrombocytes				
			0.01	Lymphocytes				
5000	0.05–0.02	Kidney weight	0.02–0.01	SGOT	0.05–0.02	SGPT	0.001	Glucose
	0.01–0.001	Alk. phosph.	0.01–0.001	SGPT	0.01–0.001	Alk. phosph.	0.001	Haemoglobin
	0.01–0.001	Haematocrit	0.02–0.01	Glucose	0.01–0.001	Neutrophil. segm.	0.05	Thrombocytes
	0.01–0.001	Neutrophil. segm.	0.001	Haemoglobin			0.01–0.001	Serum creatinine
			0.01–0.001	Serum creatinine			0.01	Lymphocytes
			0.01–0.001	Eosinophils				
			0.05–0.02	Lymphocytes				

1) Laboratory data available for evaluation: Weights of body, liver, heart, lung, kidney, testicles; SGOT, SGPT, glucose, alk. phosphatase, serum creatinine, haemoglobin, red cells, haematocrit, thrombocytes, white cells, banded neutrophils, segmented neutrophils, eosinophils, lymphocytes, monocytes, basophils, immatures.

Table 9

Statistical evaluation of laboratory results[1]). Differentiation for sex.
Significant differences between the values for treated and controls were obtained for the following pairs

Dose mg/kg/day p. o.	ε Aminocaproic acid: Sex	Figure for treated group: Higher than controls	Sex	Lower than controls	AMCHA: Sex	Figure for treated group: Higher than controls	Sex	Lower than controls
500	F	Body weight			F	Body weight	F	Haemoglobin
	FM	Liver weight			M	Liver weight	M	Thrombocytes
	M	Heart weight			M	Kidney weight		
	FM	Kidney weight			M	Testicle weight		
	FM	Red cells			M	SGPT		
	F	White cells			F	Neutrophil. segm.		
2,500	FM	Liver weight	F	SGOT	F	Lung weight	M	Haematocrit
	M	Heart weight	F	SGPT	M	Kidney weight	M	Thrombocytes
	FM	Kidney weight	FM	Glucose	M	SGPT	M	Eosinophils
	F	Alk. phosph.	M	Haemoglobin	FM	Alk. phosph.		
	M	Red cells	M	Haematocrit				
	F	Neutrophil. segm.	F	Lymphocytes				
5,000	FM	Liver weight	F	SGOT	F	Lung weight	M	Body weight
	F	Heart weight	F	SGPT	M	SGPT	FM	Glucose
	F	Lung weight	F	Glucose	M	Alk. phosph.	FM	Haemoglobin
	FM	Kidney weight	F	Serum creatinine	M	White cells	F	White cells
	M	Alk. phosph.	M	Lymphocytes	FM	Neutrophil. segm.	FM	Haematocrit
	F	Red cells					F	Thrombocytes
	FM	Neutrophil. segm.					F	Serum creatinine
							M	Lymphocytes

[1]) Laboratory data available for evaluation Weights of body liver heart, lung, kidney testicles SGOT SGPT glucose, alk. phosphatase serum creatinine, haemoglobin, red cells, haematocrit, thrombocytes, white cells, banded neutrophils, segmented neutrophils, eosinophils, lymphocytes, monocytes, basophils, immatures.

Table 10

Evaluation of anti-anaphylactic effect.

Twenty white mice per dose group Mg/kg/day p. o. fo two or f ur weeks 50 and 500.

Active AMCHA isomer mg/kg/day p. o.	Medication period	Mortality within 1 h after challenge
0 (controls)	–	14/20
50	2 weeks	10/20
500		10/20
50	3 weeks	10/20
500	"	16/20

DUBBER, MCNICOL & DOUGLAS (1964b) gave 200 mg of the active AMCHA isomer orally to volunteers. Demonstrable antifibrinolytic effect was observed at 15 minutes after administration. A maximum effect was noted at 2 hours and maintained up to 5 hours. At 12 hours a raised antifibrinolytic effect was still present, equivalent to the effect produced after about 4 hours by 2 grams of aminocaproic acid.

The acute toxicity studies reveal no problems in the use of any of the drugs tested all results give a picture of compounds without dramatic toxic effects.

In the rigorous two-generation test described, a decreased pregnancy rate and developmental disturbances were found with aminocaproic acid at the lowest dose tested corresponding to a high clinical dose. With increasing doses this trend continues, and no pregnancy occurs at a daily dose corresponding to about ten times the highest clinical dose recommended. AMCHA affects conception and foetal development considerably less. It should be noted that the two drugs have been administered at the same dose level, although AMCHA is considerably more potent than aminocaproic acid in antifibrinolytic activity

The results of the chronic toxicity studies, both in pregnant and non-pregnant rats, confirm the results on acute toxicity namely that both aminocaproic acid and AMCHA display a low degree of toxicity In the pregnant rat groups nothing remarkable occurred after administering AMCHA in doses up to and including 5000 mg/kg p o daily Aminocaproic acid under the same conditions produced signs of dehydration.

Application of results from animal two-generation studies to the clinic is difficult, especially with the limited experience still available. Comparison of the two drugs, however shows AMCHA to interfere less than aminocaproic acid with the variables studied As far as aminocaproic acid is concerned, caution must be applied in using it on women of childbearing age.

When given for three months to groups of ten male and ten female rats at each dose level, statistically significant differences from controls were reached in the large amount of laboratory results, but no tissue damage could be found.

Aminocaproic acid caused an unusual pattern of effects it significantly decreased serum creatinine at all dose levels and at the higher levels also affected the SGOT and SGPT It is not possible to decide if this reflects any effect on kidney function or any kind of improved protein utilization. A slight anaemia seemed to be a result of Aminocaproic acid administra tion at the higher dose levels

The results of AMCHA administration at about ten to about two hundred times the expected clinical dose level were of a more commonly found type. Increase in SGPT and alkaline phosphatase is the rule rather than the exception after drug administration. The increase on AMCHA medication is moderate. The average control value, 143 units, is increased to 171 192, and 169 respectively at the three AMCHA dose levels The corresponding average figures for alkaline phosphatase are 11 (for controls), 20 and 19 respectively

In the AMCHA high dose groups also signs of anaemia were recorded.

When the laboratory figures are broken down for sex, thereby reducing the group size from twenty to ten animals, other significant differences from controls came to light. Some of these differences substantiate the conclusions drawn from the undifferentiated material, of which some, especially in the aminocaproic acid group, are not easily explained. The significant increase in body and organ weights seen in the 500 mg/kg/day female groups stresses the acceptability of aminocaproic acid and AMCHA by animals.

The anti-anaphylactic properties of aminocaproic acid have been discussed on the basis of experimental and clinical results. In a test system similar to the one described here, we had found earlier that aminocaproic acid, given in doses up to 100 mg/kg intravenously at the time of challenge, did not influence the anaphylactic reaction, as judged by mortality rates. In an experiment of the same type, AMCHA shared with aminocaproic acid this lack of protective effect.

As a result of the above data, clinical investigations of the active AMCHA isomer have been initiated.

Summary

1 p-Aminomethylcyclohexane carboxylic acid (AMCHA), originally studied because of its antifibrinolytic properties, has been found to exist as two stereoisomers, of which only one possesses antifibrinolytic activ

ε Aminocaproic acid (EACA), an established antifibrinolytic agent, has been used throughout the study as reference substance.

2. *In vitro* studies on inhibition of streptokinase-induced lysis, expressed as the amount of a drug necessary for 50 / reduction of lytic activity showed the active AMCHA isomer to be twenty times more potent than aminocaproic acid.

3 The two AMCHA isomers and aminocaproic acid displayed similar urinary excretion patterns.

4 Results from up to three months medication of rats show a low degree of toxicity No tissue damage was found

5 Studies of conception and foetal development in the rat demonstrated a decrease in pregnancy rate after increasing doses of aminocaproic acid AMCHA did not influence the test system to the same degree.

6. No anti-anaphylactic effect of either compound was found.

7 The active AMCHA isomer appears safe for clinical use.

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Intestinal Absorption of Riboflavin by Man

By

Bitten Stripp

(Received January 29 1965)

During unpublished experiments with riboflavin SØREN NØRGAARD RASMUSSEN in our laboratory noticed that an increase in oral intake of riboflavin phosphate above 100 mg did not lead to an increase in its urinary excretion. LANE *et al* (1958) in experiments with riboflavin antagonists presented results indicating that the percentage of an oral dose of riboflavin appearing in the urine decreased with increased dose These facts suggest that the intestinal absorption of riboflavin is restricted to a fixed amount Little is known about intestinal absorption of riboflavin, although many investigations have been made to establish the daily requirement of the vitamin. In our study some problems of its absorption have been investigated. After different oral doses of riboflavin or flavin mononucleotide = riboflavin phosphate (= FMN) the concentrations of free riboflavin, FMN and flavin-adenine-dinucleotide (FAD) in plasma and erythrocytes were followed, and the amounts excreted in the urine were determined.

Methods

Six normal adult males, on an unrestricted diet, were used as experimental subjects. The experiments were begun between 8 and 10 a.m. The sodium salt of FMN in doses from 5 to 500 mg dissolved in about 30 ml of water or riboflavin suspended in about 300 ml of water was given orally in single or divided doses. Intravenously 84 mg of riboflavin dissolved in 1000 ml 0.9 % NaCl were administered as a drip infusion. Blood samples were taken immediately before, at ½–1 hourly intervals up to and including 8 hours after the administration and after 24 hours. Generally 0.5 ml blood was taken from the lobe of the ear into a small heparinized tube occasionally blood withdrawn from the cubital vein was used. The urinary output of riboflavin after administering the drug was determined as the total excretion in 24 hours corrected

for the basal excretion. The basal excretion expressed as *µg riboflavin excreted per minute* was determined once for each person as an average of three 24 hours' urinary output. The urine immediately before each experiment was collected for a period of 8–12 hours, in order to check that the excretion was at the normal level. The urine and blood samples were kept in the dark for analysis within 24 hours.

Analytical principle.

Total riboflavin, free riboflavin, FMN and FAD were determined in plasma, erythrocytes and urine by a modification of the fluorometric method of (BURCH *et al.* 1948). Riboflavin is determined as the difference in fluorescence between a direct reading and after addition of $Na_2S_2O_4$, which reduces riboflavin, FMN and FAD to non-fluorescent compounds.

The determination of total riboflavin and separation of the contents of free riboflavin, FMN and FAD were based on the facts given below

At pH 6.6, equimolar concentrations of FMN and riboflavin have the same intensity of fluorescence, whereas FAD has a fluorescence intensity corresponding to only 15 % of that of riboflavin (BURCH *et al.* 1948). FAD is completely hydrolysed to FMN in 10 % trichloracetic acid in 20 hours at 38°C (BESSEY *et al.* 1949). By distribution between β-phenylethanol and the aqueous phase (pH 6.6) the distribution coefficients at room temperature for riboflavin, FMN and FAD were found to be 3.06, 0.018 and 0.042, respectively

Materials.

Riboflavin U.S.P

Riboflavin mononucleotide (FMN) as sodium salt (Sigma) Flavin adenine dinucleotide (FAD) (Sigma)

Trichloroacetic acid 10 % (Fe^{+++} free with redistilled water)

β-phenylethanol (saturated with redistilled water)

Sodium hydrosulphite, $Na_2S_2O_4$ (Merck)

Sodium bicarbonate, $NaHCO_3$ (Merck)

Potassium phosphate, K_2HPO_4 (Baker)

Chloroform (saturated with redistilled water).

Procedure

All preparations were carried out in the dark or in a darkened room equipped with red light.

Determination of total riboflavin = free riboflavin + FMN + FAD in plasma.

Separate blood cells from plasma by centrifugation. Blow 100 µl plasma into 1.0 ml of 10 % trichloroacetic acid, mix well, and allow to stand for 15 minutes. Centrifuge for 10 minutes (3000 r p.m.), transfer the clear supernatant to a test tube, close with a cork stopper and hydrolyse for 20 hours at 38°C. Measure the fluorescence at 800 µl, neutralized with 800 µl M K_2HPO_4, on a "Photovolt" filter fluorometer with "Hg2 3" as primary and "B-540" as secondary filter Carefully add 50 µl of 10 % $Na_2S_2O_4$ in 5 % $NaHCO_3$, and measure the fluorescence again. A freshly prepared standard solution of riboflavin (10 ng/ml) in 10 % trichloroacetic acid neutralized with phosphate buffer as described above, is measured in the fluorometer in order to check the readings from day to day However a variation of only 2 5 % was found.

Calculation.

The difference between the two readings before and after addition of $Na_2S_2O_4$ to the test solution (a) is multiplied by 22 (dilution factor) and corrected for the readings of the standard solution (b). Thus, the concentration of total riboflavin in plasma is

$$\frac{a \quad 22 \quad 10}{b} \text{ ng/ml}$$

Determination of free riboflavin, FMN and FAD in plasma or erythrocytes.

Blow 1.0 ml plasma or erythrocyte suspension into 10.0 ml trichloroacetic acid at 0° Immediately after centrifugation neutralize 6.0 ml of the supernatant with 6.0 ml M K_2HPO_4 at room temperature, and measure the fluorescence on a 5 ml portion treated with 0.5 ml $Na_2S_2O_4$ (A).

Shake 7.0 ml of the neutralized solution with 10 0 ml β-phenylethanol for two minutes. Centrifuge for 5 minutes, transfer 6 ml of the lower aqueous layer to 2 ml chloroform, shake for ½ minute. After complete separation, remove the upper layer *for measurement of the apparent riboflavin (C).*

Determine total riboflavin (B) on the trichloroacetic acid supernatant after 24 hours, as described above.

Calculation.

If 7.0 ml neutralized solution are extracted, as described, with 10.0 ml β-phenylethanol, and the distribution coefficients of 3 06, 0.018, and 0.042 are used, 18.5% *riboflavin, 97.5% FMN and 94.4% FAD will be left in the aqueous phase. The FAD* will, as mentioned above, show a fluorescence only 15 / that of the equivalent amount of riboflavin. The apparent riboflavin determined as described above will thus represent

$$A = \text{free riboflavin} + \text{FMN} + 0.15 \text{ FAD}$$
$$B = \text{free riboflavin} + \text{FMN} + \text{FAD}$$
$$C = 0.185 \text{ free riboflavin} + 0.975 \text{ FMN} + 0.14 \text{ FAD}$$

From these equations it may be calculated that

$$1)\ \text{FAD} = \frac{B - A}{0.85}$$
$$2)\ \text{FMN} = 1.27\,C - 0.169\,B - 0.0672\,A$$
$$3)\ \text{free riboflavin} = B - \text{FMN} - \text{FAD}$$

in which FMN and FAD are calculated as riboflavin.

Determination of riboflavin in urine

The urine from 2 persons before and after administration of FMN was examined. Thin layer chromatography (pyridine/glacial acetic acid/water 10/1/40) showed no FAD and extraction of neutralized solution of urine with β-phenylethanol showed negligible amounts of FMN The determination of riboflavin in urine was, therefore, simplified.

Add 5 or 10 μl of urine to 5 0 ml buffer solution pH 6.6 (equal volumes of M-K_2HPO$_4$ and 10% trichloroacetic acid), measure the fluorescence and compare readings with those of the standard riboflavin solution to calculate the of riboflavin in the urine samples.

Table 1

Recovery of riboflavin, FMN or FAD added to plasma *in vitro*.
The values in this and in the next table and figures are the means of duplicate analyses. The average difference between two corresponding results was found for plasma 1.9 ng/ml, for urine 0.20 μg/ml.

Added to 100 ml plasma	Total riboflavin recovered ng/ml	%	Free riboflavin recovered ng/ml	%	FAD recovered ng/ml	%	FMN recovered ng/ml	%
0	68.1		28.1		27 7		1..3	
6.2 μg riboflavin	128.5	98	77 7	80				
6.1 μg FAD	124.0	93			79 9	85		
4 4 μg FMN	110.3	96					52.1	91

Recovery

Recovery of riboflavin, FMN and FAD added to plasma *in vitro* is shown in table 1 Recovery of 830 μg riboflavin added to 100 ml urine was 92%.

Results

Normal values for riboflavin and its derivatives in plasma and in urine are shown in table 2. The concentration of total riboflavin in plasma was 52 ng/ml (range 36–81 ng/ml) of which FAD constituted approximately two thirds. The average concentration of riboflavin in urine was 1 4 μg/ml (range 0.2–3.5)

The concentrations of free riboflavin, FMN and FAD in plasma and

Table 2

Average concentrations of total riboflavin, riboflavin, FMN and FAD in plasma and urine of 6 normal persons.

	Plasma ng/ml	Urine μg/ml
Riboflavin n = 12	13 (3–22)	–
FMN n = 12	6 (1 20)	
FAD n = 12	33 (17 74)	
Total riboflavin	52 (36–81)	1 4 (0.2 3.5)

n = number of observations highest and lowest values in brackets.

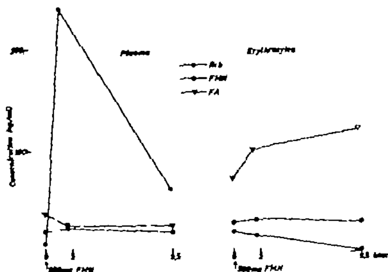


Fig 1 Concentration of free riboflavi (Rib.), flavin mononucleotide (FMN), and flavin-adenine dinucleotide (FAD) in plasma and erythrocytes after oral administration of 500 mg FMN (flavin mononucleotide). Abscissa hours after administration. Ordinate ng per ml plasma or erythrocytes of riboflavin (—●—), FMN (—○—), FAD (—▽—).

erythrocytes after administration of 500 mg FMN are shown in fig. 1 The concentration of free riboflavin in plasma increased from 6 to 245 ng/ml during the first two hours, but the concentrations of FMN and FAD remained almost unchanged. In the red blood cells the concentrations of free riboflavin and FMN were practically unchanged, whereas the concentrations of FAD rose from 70 to 120 ng/ml.

Fig. 2 shows the urinary excretion after administering various doses of FMN From 0 to 20 mg there was approximate linearity between the dose

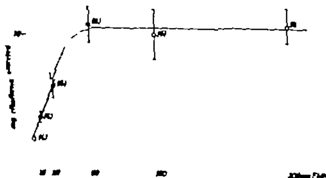


Fig. 2. Excretion in the urine after oral administration of various doses of FMN (flavin mononucleotide). Abscissa Doses of FMN in mg. Ordinate 24 hours urinary output of riboflavin in mg (corrected for basal excretion). Values in brackets are numbers of experimental subjects. The vertical lines represent standard errors of the means.

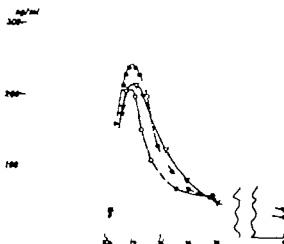


Fig. 3. Concentration of total riboflavin in plasma (person E) after oral administration of 50, 100 or 500 mg FMN (flavin mononucleotide). Abscissa time of day in hours, arrow: time of take. Ordinate concentratio in ng per ml plasma. (—●—) 500 mg. (—○—) 100 mg. (—▽—) 50 mg. Total excretion after 500 mg 14.7 mg. 100 mg 10.3 mg. 50 mg 14.1 mg.

and the amount excreted. An increase in the dose above 50 mg did not cause any further increase in the excretion.

Fig. 3 illustrates the concentration of total riboflavin in plasma as a function of the time after various single oral doses of FMN The highest value (240 ng/ml plasma) was observed 1½ hour after the administration and was independent of the dose.

The results of a similar experiment in another person, F are shown in fig. 4 only there riboflavin was administered instead of FMN The concentration of total riboflavin in plasma reached its maximum after the same interval as after administration of FMN the value again being independent of the dose. Fractionation of the plasma showed an increase in the concentration only of free riboflavin.

The urinary excretions of riboflavin were 17.0, 17.9 and 14.0 mg after administering 100 and 500 mg riboflavin and 200 mg FMN respectively

Administering 2.5 g dried, defatted hog stomach mucosa plus 500 mg riboflavin to person F yielded the same results as shown in fig. 4 the excretion being 18.7 mg.

The effect of administering 4 doses of 50 mg FMN at intervals of 90 minutes is shown in fig. 5 The time during which total riboflavin concentration in the plasma was at peak level was prolonged, and the excretion of riboflavin was slightly over 100 / more than that found after a single dose of 50 or 500 mg.

After intravenous administration of riboflavin, the plasma total riboflavin concentration was 6 times higher than after an oral dose of

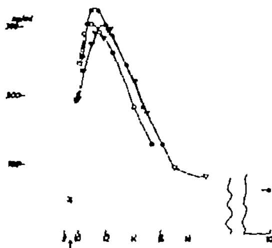


Fig. 4. Concentration of total riboflavin in plasma (person F) after oral administration of 100 or 500 mg riboflavin or 200 mg FMN (flavin mononucleotide). Abscissa time of day in hours arrow time of intake. Ordinate concentration in ng per ml plasma. (—●—) 100 mg riboflavin, (—▽—) 500 mg riboflavin, (—○—) 200 mg FMN.
Total excretion after 500 *mg riboflavin* 17.9 *mg*, 100 *mg riboflavin* 17.0 *mg*, 200 mg FMN 14.0 mg.

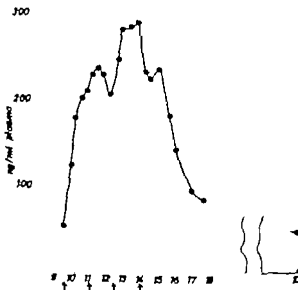


Fig. 5. Concentration of total riboflavin in plasma (person E) after administration of 200 mg FMN (flavin mononucleotide) given as fractioned doses of 50 mg. Abscissa time of day in hours, arrow intake. Ordinate concentration in ng per ml plasma. Total excretion 32.0 mg.

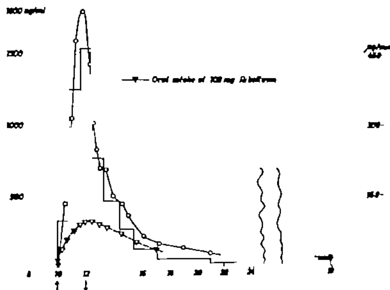


Fig. 6. Concentration of total riboflavin in plasma after intravenous infusion (between arrows) of 84 mg (—○—) or oral intake of 100 mg riboflavin (—∇—). Excretion of riboflavin in urine expressed in µg per minute is indicated by the columns. Total excretion 81 mg (97 %) after oral intake 17.0 mg.

100 mg riboflavin (fig. 6) The excretion of riboflavin in urine was found to be 81 mg, 97 % of the dose administered

Discussion

The results show that not more than 14–18 mg riboflavin is excreted in the urine, independent of the oral dose when this is increased above 50 mg in single doses. Up to doses of 20 mg FMN the excretion of riboflavin in urine was found to be in agreement with the results of MORRISON & CAMPBELL (1960), who showed that riboflavin excretion, in contrast with excretion of thiamine, was proportional to the oral intake their doses, however did not exceed 20 mg. When the doses were increased from 50 to 500 mg. no further increase in urinary riboflavin was found indeed, small decreases were observed These results are in agreement with those of LANE *et al* (1958) who presented figures indicating that the percentage in the urine after an oral dose from 50 to 500 mg decreased with increasing dose. After oral intake of FMN the rise in concentration of FAD in erythrocytes within 9 hours was only 20 % that of free riboflavin in plasma within 2 hours therefore, only the plasma was examined. No fractiona tion was necessary since free riboflavin was the only fraction increased.

The highest plasma concentration of riboflavin was found to be independent of the increase in oral intake above 50 mg. Intravenous administration of riboflavin gave a much higher peak level of plasma riboflavin and almost complete excretion in the urine, showing that riboflavin is not retained in the organism. Thus the output in urine is a fairly good measure of the amount absorbed and the limited excretion therefore suggests a limited absorption. It is unlikely that destruction in the small intestine explains the incomplete absorption, as the urinary excretion of riboflavin after doses from 0 to 20 mg is consistently 30–50 % of the given amount, and thus increases with the dose. Because of the amount of urinary excretion it is therefore assumed that the absorption of riboflavin or FMN cannot exceed approx. 18 mg after a single dose.

Further studies have been initiated in order to investigate the circumstances of the absorption.

YAGI (1954) *showed that subcutaneous injection of riboflavin into rats* caused an increase of FMN and free riboflavin in the mucosa of the small intestine. The increase in FMN was explained as a result of a phosphorylation of riboflavin in the small intestine. A synthesis of FMN made from riboflavin and a phosphate donor by action of the phosphomonoesterase from the small intestinal mucosa has been effected by YAGI & OCUDA (1958) *in vitro*. These *in vivo* and *in vitro* results also suggest that phosphorylation takes place in small intestine *in vivo* but do not indicate whether the process is necessary for the absorption of riboflavin. The fact that only free riboflavin is increased in the blood after administration of FMN indicates that a dephosphorylation of FMN occurs somewhere before it reaches the blood stream.

SPENCER & ZAMCHECK (1961) concluded from *in vitro* experiments with *small intestine from rats that riboflavin is absorbed by simple physical* diffusion. This is not in agreement with our results. If diffusion were the only path of absorption, the plasma riboflavin level and the excretion in the urine would rise in proportion *to the increase in oral intake.*

Since gastric mucosa was shown to have no effect on the amount of riboflavin excreted or the peak level in plasma, *the absorption of riboflavin* cannot be analogous to what is known of the absorption of cyanocobalamin (vitamin B_{12}). The facts that the essential absorption of a single dose is almost complete within 6 hours and that increased absorption is seen when 200 mg is given in divided doses may indicate that only part of the small intestine – probably the proximal end – can absorb riboflavin. It also suggests that the absorptive capacity returns to normal values within a few hours.

It is concluded that only a limited amount of riboflavin can be absorbed from the intestinal tract.

Summary

1 After oral administration of riboflavin or *flavin mononucleotide to* human subjects, a rise in the concentration of free riboflavin occurred only in plasma, and only riboflavin was excreted in the urine.

2. An oral intake of riboflavin or flavin mononucleotide above 50 mg *caused no further increase in the urinary excretion of riboflavin.*

3 The "absorption curves" of riboflavin in plasma after oral intake of riboflavin or flavin mononucleotide in amounts from 50 to 500 mg have quantitatively and qualitatively a shape independent of the dose.

4 Recovery in the urine of riboflavin administered intravenously was 97 %.

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Localization of Catecholamine Uptake in Rat Brain Slices

By

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By means of the histochemical fluorescence method of FALCK & HILLARP recently described in detail by DAHLSTRÖM & FUXE (1964) and by NORBERG & HAMBERGER (1964), it has been demonstrated that not only the terminals but also the entire peripheral adrenergic neuron have a specific mechanism for uptake and concentration of noradrenaline (NA) and related amines. This mechanism is localized at the level of the cell membrane and is blocked by certain drugs (*i. e* cocaine, chlorpromazine and desmethylimipramine), but it is insensitive to reserpine (HAMBERGER *et al* 1964 HILLARP & MALMFORS 1964 MALMFORS 1965) The important problem whether the recently discovered NA and dopamine neurons in the brain, which in all probability use these amines as transmitters (see DAHLSTRÖM & FUXE 1964 FUXE 1965a & b) also possess such a mechanism is difficult to study *in vivo* as a barrier mechanism for the uptake of catecholamines from the blood has been demonstrated (WEIL MALHERBE, WHITBY & AXELROD 1961) Labelled catecholamines have been shown to be taken up and concentrated by brain slices *in vitro* (DENGLER *et al.* 1962), but the cellular localization of this uptake has not been elucidated. It has, however been found that central nerve terminals of the adrenergic type in the median eminence, which lacks the above-mentioned blood-brain barrier readily take up and accumulate exogenous NA *in vivo* (FUXE & HILLARP 1964). – In our study the *in vitro* technique was combined with the specific fluorescence method for certain monoamines.

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Material and Methods

Male albino rats (Sprague-Dawley) were pretreated with reserpine (5 mg/kg, i. p., 12–18 hours) to deplete the brain stores of their catecholamines. Nialamide (100 mg/kg, i. p.) was administered to some of the animals 2–4 hours before killing them by bleeding out under light ether anaesthesia. Slices, prepared by the technique of McILWAIN & RODNIGHT (1962), were taken from the cerebral cortex, brain stem and, for comparison and control, the vas deferens and were incubated at 37° in a modified Krebs-bicarbonate medium (AXELROD *et al.* 1962) containing glucose (10^{-2}M). The amines (—)-NA or (—)-α-methyl NA were added at the beginning of incubation or after preincubation for 15 minutes, and the incubation time was 20 minutes. After incubation the slices were immediately frozen, freeze-dried, treated with formaldehyde gas, embedded and sectioned for fluorescence microscopy (for details see DAHLSTRÖM & FUXE 1964 NORBERG & HAMBERGER 1964). The NA and α-methyl NA taken up are converted during the formaldehyde treatment without significant diffusion into intensely fluorescent green to yellow-green 3 4-dihydroisoquinolines (CORRODI & HILLARP 1963 1964).

Results and Discussion

The synaptic terminals of the central catecholamine neurons and the peripheral adrenergic terminals have a characteristic appearance, showing abundant enlargements, varicosities, which represent the presynaptic structures specialized for synthesis, storage and release of the catecholamines (see DAHLSTRÖM & FUXE 1964 MALMFORS 1964 NORBERG & HAMBERGER 1964 FUXE 1965a) Control slices (from reserpine or reserpine and nialamide treated animals) directly frozen or incubated without amines showed that the reserpine treatment had caused total depletion of the endogenous catecholamines stored in the terminals. Incubation in a medium containing α methyl NA resulted in the appearance of typical adrenergic synaptic terminals, which according to the concentration of the amine (0 01–1 μg/ml), showed weak to strong green to yellow-green fluorescence (fig. 1) These terminals seemed to have the same characteristic appearance and distribution as those observed in the brain from normal animals, and no such terminals were seen in brain regions that normally lack catecholamine terminals. There is thus little doubt that the observed terminals are identical with the synaptic terminals of the specific catecholamine neurons In some parts of the cerebral cortex a fairly dense network of thin fluorescent terminals could be observed. Both in the cortex and brain stem there were also smooth fibres without varicosities, which showed about the same fluorescence as the varicose terminals and mostly ran in fibre bundles (fig. 2) There is little doubt that these fibres are non-terminal axons belonging to catecholamine neurons. These axons normally contain such low concentrations of catecholamines that they exhibit no or only weak fluorescence (*cf* DAHLSTRÖM & FUXE 1964 FUXE 1965b).

Fig. 1 Lower brain stem from reserpine-pretreated rat incubated in a medium containing α-methyl NA (1 μg/ml). A high accumulation of the amine can be seen in numerous adrenergic terminals. Note the characteristic appearance of the terminals with brilliantly fluorescent enlargements, varicosities and the thin segments in between. 440 ×



Fig. 2. Lower brain stem from reserpine-pretreated rat incubated in a medium containing α-methyl NA (1 μg/ml). Bundles of non-terminal axons that have accumulated the amine can be seen. T the *right* also single fluorescent non-terminal axons can be seen. Note the smooth appearance of these axons, easily distinguished from the terminals. 260



Fig. 3. Cortex from reserpine-pretreated rat incubated in medium containing α-methyl NA (10 μg/ml). The walls of the capillaries, and especially certain cells, probably the pericytes, accumulate the amine to high levels. 180 ×

The adrenergic terminals and non terminal axons in slices from the vas deferens always showed a somewhat higher amine uptake than those in the brain slices, probably owing to the greater thickness of these structures in the former

The terminals in slices from reserpine and nialamide treated animals showed a similar uptake and accumulation of both NA and α-methyl NA. However if the animals were not pretreated with the potent monoamine oxidase (MAO) inhibitor nialamide, there was almost no accumulation with 1 μg/ml NA in the medium and only a weak to moderate accumulation with 10 μg/ml. On the other hand, the α-methylated amine, which is not readily attacked by MAO accumulated to about the same degree whether the animals were pretreated with nialamide or not. These findings support the view that MAO is present in the central catecholamine terminals (see CARLSSON & HILLARP 1962 CARLSSON 1965 DAHLSTRÖM FUXE & HILLARP 1965), as seems to be true for the peripheral adrenergic terminals (HILLARP & MALMFORS 1964 NORBERG & HAMBERGER 1964 MALMFORS 1965 NORBERG 1965)

Our experiments show that the synaptic terminals and axons of the central catecholamine neurons – like those of the peripheral adrenergic neurons – have an efficient mechanism for uptake and concentration of exogenous NA Judging by the fluorescence intensity (cf NORBERG & HAMBERGER 1964 MALMFORS 1965), amine levels in the order of 1 000–10 000 μg/g wet weight may be attained in the terminals by incubation in a medium containing 1 μg/ml of NA or α methyl NA. Since there is strong evidence that reserpine blocks the uptake and storage mechanisms of the amine storage granules (see DAHLSTRÖM, FUXE & HILLARP 1965 for references), the amines taken up accumulate in all probability in the axoplasm without co-operation of the storage granules. The fact that the non terminal axons, which on the basis of their normally low catecholamine content may be assumed to contain only few storage granules (HAMBERGER et al 1964), also accumulate exogenous amines to high levels strongly supports this view The central catecholamine neurons, consequently have a mechanism for uptake and concentration of NA that seems to be localized at the level of the cell membrane.

In the brain slices not only the catecholamine neurons, but also cells of the capillary wall (but not of the larger vessels) showed the capacity to take up and concentrate NA and α-methyl NA to high levels (fig. 3). In appearance and localization these cells seem to be identical with pericytes (cf LANDERS et al 1962 WOLFF 1962) No accumulation has, however been observed in the cells on intravenous administration of the amines (FUXE & HILLARP 1964). If the animals were not pretreated with nialamide, only α methyl NA but not NA accumulated in vitro, indicating

MAO activity in the pericytes. The possibility then arises that the pericytes or the capillary endothelium or both may exert an important function in the removal from and inactivation of catecholamines in the brain.

Our experiments show that the technique used is of value for direct studies of neuronal and extraneuronal uptake of catecholamines in the brain. The technique also opens new possibilities for studying the effects of drugs on the uptake and storage mechanisms. Thus, for instance, it was found that cocaine (10^{-4} to 10^{-3} M) strongly inhibited the neuronal uptake of NA.

Summary

The *in vitro* uptake of noradrenaline (NA) and α-methyl NA by rat brain was studied directly at the cellular level by a sensitive histochemical fluorescence method. The animals were pretreated with reserpine to deplete the brain stores of their endogenous monoamines. The axons and synaptic terminals of the central catecholamine neurons were found to be capable of accumulating the amines to high levels showing that the central, like the peripheral, adrenergic neurons have an efficient reserpine-insensitive uptake and concentration mechanism for NA, which seems to be localized at the cell membrane. Cocaine strongly inhibited the neuronal uptake.

Certain cells, probably pericytes, in the walls of the capillaries also showed an efficient mechanism for amine uptake and seem to possess high monoamine oxidase activity. This suggests that they may have an important function in removal and inactivation of catecholamines in the brain.

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Influence of Antirheumatic Agents on the Release of Histamine from Rat Peritoneal Mast Cells After an Antigen-Antibody Reaction

By

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The role of histamine in various allergic or anaphylactic reactions is of great interest, as histamine is one of the mediators of the allergic and inflammatory response Thus, the release of histamine from sensitized organs has been demonstrated *in vivo* as well as *in vitro* and histamine has been detected in the blood or perfusion fluid after the administration of antigen (TELFORD & WEST 1963)

Most of the histamine in the body is stored in mast cells (TELFORD & WEST 1963) During the anaphylactic reaction the antigen-antibody reaction damages the mast cells, and histamine is released. This phenomenon has been elucidated by KELLER (1962a & b) on isolated peritoneal mast cells from the rat.

The immunological reaction is presumably an important factor in eliciting rheumatoid arthritis and collagen diseases (MACKAY & BURNET 1964) Cortisone inhibits the immunological reaction. This is observed in animal experiments in which cortisone medication inhibits antibody formation (GERMUTH 1956 FISCHEL *et al* 1952 MACKAY & BURNET 1964) and prolongs the survival time of skin homografts (MEDAWAR & SPARROW 1956). It is doubtful whether cortisone inhibits the union of antigen and antibody but it may reduce possibly the sensitivity of the cells to the antigen-antibody action (GOODMAN & GILMAN 1955 SOLLMANN 1957 MACKAY & BURNET 1964)

Our study was designed to investigate the effect of some glucocorticosteroids as well as of non-steroidal antirheumatic agents and other sub-

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stances on the release of histamine after an antigen-antibody reaction in a suspension of peritoneal mast cells from sensitized rats. It comprises treatment of the rats with antirheumatic agents towards the end of the sensitization period, as well as *in vitro* experiments in which the substance was added direct to the mast-cell suspension before adding the antigen.

Experiments and methods

Adult male albino rats (Wistar) weighing about 120 g were used.

Doses and injection solutions The substances were administered subcutaneously in the quantities shown in table 2. When the substance was soluble in water it was administered as an aqueous solution otherwise it was suspended in a mixture of 1 part of Acacia Mucilage (U.S.P 47) and 6 parts of water In either event the dosage was 2.00 ml/kg.

Sensitization of the rats The rats were sensitized to horse serum by a subcutaneous injection of 1 0 ml horse serum (inactivated by heating for half an hour at 56°) mixed with 1 0 ml (10^{10} bacilli) pertussis vaccine. At the end of 4 days the treatment was repeated with 0.5 ml horse serum and 1 0 ml pertussis vaccine. The rats were used as sensitized animals (HÖGBERG & UVNÄS 1958) in experiments 3 weeks after the first treatment. Their body weights now ranged from 150 to 200 g.

In vitro experiments Into each sensitized rat 18 ml of a modified Tyrode solution, heated to 38°C, were injected intraperitoneally this is a buffered solution (pH 7.3) containing $1\,44 \times 10^{-1}$ M NaCl, 3×10^{-3} M KCl, 8×10^{-4} M-$CaCl_2$, 2 H_2O 8×10^{-3} M Na_2HPO_4, 2 H_2O and 3×10^{-3} M-KH_2PO_4. The rat was killed by bleeding from the carotid arteries 30 seconds after the injection. The abdominal wall was opened, and approximately 12 ml of the injected solution, now containing mast cells, were removed from the peritoneal cavity The fluids were pooled, and 8.00 ml samples of the mast-cell pool were removed. To 3 of these samples (control samples) 2.00 ml modified Tyrode solution were added, and the substances listed in table 1 dissolved in 2.00 ml modified Tyrode solution, were added to the other samples (test samples). The concentrations given in the table refer to the final volume of 10 ml. To all samples 0.50 ml horse serum (antigen) was added, and the mixture was incubated at 38° for 30 min. The amounts of histamine released from the mast cells by the antigen-antibody reaction were determined after centrifugation (3400 × g for 15 min.) in the mast-cell free supernatant which was decanted. Next were added 5.0 ml 15% (w/v) trichloracetic acid and 1.50 ml 4 N sodium acetate, 3 H_2O. The mixture was transferred to a Decalso column, and the adsorbed histamine was eluted, coupled and measured spectrophotometrically as previously described (NORN 1965). The contents of released histamine in the samples were corrected for the amounts liberated by the mechanical manipulation of the mast-cell suspension. This quantity was determined in 3 samples of 8.00 ml from the mast-cell pool carried through the analysis in parallel with the control samples, but incubated without the addition of horse serum. The total amounts of histamine in the samples removed from the mast-cell pool were determined in 3 samples of 8.00 ml. To release the intracellular histamine, 4.0 ml 15 (w/v) trichloracetic acid were added to the removed specimens. After having stood for 20 min. the mixture was centrifuged (3400 × g for 15 min.). To the decanted fluid were added 1.2 ml 4 N sodium acetate, 3 H_2O and the mixture was transferred to a Decalso column the analysis was then continued as described above.

In vivo experiments. Half the sensitized rats were treated, during the last 3 days of the sensitization period, with a daily subcutaneous injection of one of the substances listed in table 2. The treated rats are called test rats and the untreated ones control rats. A volume of 18 ml of modified Tyrode solution (38°) was injected intraperitoneally into both test and control rats, the test group being injected 1.5 hours after the last dose of the substance to be tested. The rats were killed 30 sec. after administering the modified Tyrode solution, and the abdominal cavities were emptied of fluid. In order to obtain a sufficient quantity of peritoneal mast-cell suspension per sample, the contents from 2 rats in the test or the control group were pooled. The mixtures are designated test and control samples. From each of these samples 2 portions of 10.0 ml were removed. 0.50 ml horse serum was added to one (specimen a) and the same volume of modified Tyrode solution to the other (specimen b). Specimens a and b were incubated at 38° for 30 min. After centrifugation (3400 × g for 15 min.) the released quantities of histamine in the supernatant were determined as described under the *in vitro* experiments. The difference between released histamine in sample a and b corresponds to the released content per 10.0 ml test or control sample, as a correction has been made for the quantity released during the manipulation. The total histamine content per 10.0 ml test or control sample was calculated as the sum of the histamine contents in supernatant and sediment from specimen b. The latter content was determined as described below. The histamine was freed from the mast-cell sediment by careful shaking and standing for 15 min. with 2.5 ml 20 % (w/v) trichloracetic acid. After adding 5 ml modified Tyrode solution and 1.0 ml 4 N sodium acetate, 3 H_2O the mixture was centrifuged, and the decanted fluid was poured on to a Decalso column, and the analysis continued as already described.

Determination of mast-cell concentration. The concentration of mast cells was determined in mast-cell suspensions from control and test rats as advocated by BRAY & ASBOE (1961). Moreover the concentrations of total nucleated cells and of erythrocytes were determined in suspensions from some control rats.

Results

In vitro experiments

The substances mentioned in table 1 were added, at various concentrations, to a suspension of peritoneal mast cells from sensitized rats (test samples). After incubation with antigen the quantity of released histamine was determined. The release of histamine after incubation without previous addition of substance (control samples) is usually around 40 % of the total content of the sample. The inhibition of histamine release in the test samples, as given in table 1, was calculated according to the formula

$$\frac{(Hi_c - Hi_t) \times 100}{Hi_c - Hi_m} \%$$

in which the symbols represent the quantity of histamine released in the control sample (Hi_c), test sample (Hi_t) and mast-cell suspension without addition of substance or antigen (Hi_m). Among the substances investigated chlorprothixene and amitriptyline showed the greatest effect, a

Table 1

In vitro experiments.

Inhibitory effect of anti rheumatic agents and other substances on the release of histamine after an antigen-antibody reaction in a suspension of peritoneal mast cells from sensitized rats.

Percentage inhibition of histamine release.

Substance	Added µg substance per ml mast cell susp.					
	0.1	1.0	10	100	1000	10000
Hydrocortisone	0	0	0	–	–	
Methylprednisolone	0	0	0	–	–	–
Phenylbutazone	0	0	0	27	95	–
Sodium salicylat	0	0	0	3	74	98
Sodium-γ-resorcylate	0	0	2	16	11	77
Amidopyrine	0	0	0	0	6	95
Chlorprothixene	0	0	66	–	–	–
Amitriptyline	0	0	50	–	–	–

tion of 10 µg/ml giving a 50 % inhibition of histamine release. At higher concentrations the mast cells will be destroyed, so that histamine is released in larger quantity than in the control samples. No inhibition was obtained with hydrocortisone or methylprednisolone the solubility of these substances does not permit a study of concentrations above 10 µg/ml. Phenylbutazone and sodium salicylate showed an inhibitory effect between $\frac{1}{10}$ and $\frac{1}{100}$ and sodium-γ-resorcylate and amidopyrine an effect between $\frac{1}{100}$ and $\frac{1}{1000}$ those of chlorprothixene and amitriptyline.

In vivo experiments

Sensitized rats were treated towards the end of the sensitization period with the substances listed in table 2 (test group) From these rats, and from sensitized rats untreated with any of the substances named (control group), a suspension of peritoneal mast cells was removed and incubated *in vitro* with antigen. The released histamine was corrected for the quantity liberated during the manipulation and determined as a percentage of the total amount in the sample In the control as well as in the test samples, the total contents amounted to 8.8 µg histamine base calculated per rat (s. = 3 1) (no significant difference in contents according to the t-test $P > 0.05$) The release due to manipulation was about 15 % of the total amount in the sample. There was no significant difference in "manipulation release" between control and test samples ($P > 0.05$). The corrected release of histamine elicited by the antigen-antibody reaction varied from specimen to specimen within the control group As a rule, it was between

30 and 50 % of the total amount in the specimen, more rarely between 20 and 60 %. The day-to-day variation exceeded the variation within each day and there could be significant differences between the releases from one day to another. Accordingly the test samples were always compared with control samples for the same day.

The inhibition of histamine release in the test samples was calculated according to the formula.

$$\left(1 - \frac{Hl_{ta} - Hl_{tb}}{\text{tot } Hl_t} \times \frac{\text{tot } Hl_c}{Hl_{ca} - Hl_{cb}}\right) \times 100\ \%$$

in which Hl_{ta}, Hl_{tb}, Hl_{ca} and Hl_{cb} are the amounts of histamine released in samples a and b in the test and control samples, respectively. The expressions tot Hl_t and tot Hl_c indicate the total histamine contents in test and control samples. The percentage inhibition is recorded in table 2, which also shows the number of test and control samples. It indicates a significant decrease in histamine release.

All glucocorticosteroids inhibit histamine release, and the inhibition rises with increasing dosage. With the highest doses there was a highly significant inhibition of histamine release. Among non-steroid antirheumatics, sodium aurothiosulphate, at a dosage of 25 mg/kg, showed a highly significant inhibition of 87 %, as did phenylbutazone, at a dosage of 200 mg/kg, the inhibition being 32 %, which is slightly significant.

Cell counts and histamine values

Differential counts of the mast cell suspension from 15 sensitized control rats revealed 26 ± 4 (s.e.m.) mast cells per square mm (table 3) corresponding to 2.6 % of the total nucleated cells. Erythrocytes were found in a number of approx. 70 per square mm suspension. The mean content of total histamine was 0.8 ± 0.1 (s.e.m.) µg histamine base per ml, and the histamine content of the mast cells was, therefore, calculated as 31 µg histamine base per 10^6 mast cells, assuming that the other cells contained very little or no histamine (LAGUNOFF & BENDITT 1960).

The concentration of mast cells and the total content of histamine were determined in mast-cell suspensions from sensitized rats treated hydrocortisone, phenylbutazone or sodium aurothiosulphate (table
Neither the mast cell count per square mm test sample nor the c
total histamine per ml test sample showed a significant difference from
values for the control samples ($P > 0.05$ by t-test).

The mast-cell suspension from 15 non-sensitized and untreated showed a mast-cell concentration and a total histamine

Table 2

In vivo experiments.

Inhibitory effect of antirheumatic agents and other substances on the release of histamine after an antigen-antibody reaction in a suspension of peritoneal mast cells from sensitized rats. Significant differences from control specimens are indicated in terms of P by the t-test.

Inhibition of histamine release

Test specimens number	Control specimens number	Substance	Daily dose 3 days mg/kg	% inhibition	P
4	3	Hydrocortisone	200	60	+
14	11	Hydrocortisone	100	58	++
4	3	Hydrocortisone	50	43	
4	3	Hydrocortisone	25	18	
5	5	Fluorometholone	100	69	+
5	5	Fluorometholone	50	39	
5	5	Fluorometholone	25	7	
10	10	Methylprednisolone	5.0	75	++
5	5	Methylprednisolone	3.6	33	
5	5	Methylprednisolone	2.5	14	
5	5	Betamethasone	5.0	82	++
5	5	Betamethasone	2.5	37	
5	5	Betamethasone	1.25	34	
5	5	Betamethasone	0.63	0	
15	14	Phenylbutazone	200	32	+
4	4	Phenylbutazone	100	20	
5	5	Monophenylbutazone	400	0	
5	5	Monophenylbutazone	100	2	
5	5	Oxiphenbutazone	200	0	
5	5	Sodium aurothio-sulphate	25	87	++
5	5		5	0	
4	3	Chloroquine diphosphate	50	0	
5	5	Indomethacin[1])	2.5	0	
4	4	Sodium salicylate	200	0	
4	4	Sodium-γ-resorcylate	200	0	
5	5	Acetylsalicylic acid	150	0	
8	8	Amidopyrine	200	14	
4	3	Mepyramine	20	9	
4	4	Chlorprothixene	25	0	
4	3	Amitriptyline	25	11	

++ $P \leq 0.01$
\+ $0.05 > P > 0.01$

1) A higher dose could not be given owing to development of multiple intestinal necroses and peritonitis.

complete conformity with the findings on suspensions from sensitized control rats ($P > 0.05$).

Discussion

A suspension of peritoneal mast cells from sensitized control rats showed a mast-cell concentration of 26 ± 4 (s.e.m.) cells/mm^3 and a

Table 3

Mast cell counts and histamine values in suspensions of peritoneal mast cells from non-sensitized and sensitized rats. The latter included control as well as treated animals.

Substance	Daily dose 3 days mg/kg	Mast cells per mm³ specimen Mean ± s.e.m.	Total histamine (μg base) per ml specimen Mean ± s.e.m.
Non-sensitized control	–	35 ± 4	0.8 ± 0.1
Control	–	26 ± 4	0.8 ± 0.1
Hydrocortisone	200	26 ± 3	0.8 ± 0.1
Phenylbutazone	200	17 ± 3	0.7 ± 0.1
Sodium aurothiosulphat	25	28 ± 8	0.6 ± 0.1

total histamine content of 0.8 ± 0.1 (s.e.m.) μg base/ml. These values are in agreement with those found for non-sensitized rats. Thus no reduction of mast cells occurs in the peritoneal cavity during sensitization. In the removed mast-cell suspension from non-sensitized rats BRAY & ARSDEL (1961) found a concentration of mast cells and a total content of histamine 5 times higher than we have done. This is presumably due to a difference in the strains of rats used and the difference in their body weight or possibly to a more effective removal of cells from the peritoneal cavity. The histamine content of the mast cells was calculated by us as 31 μg histamine base/10^6 mast cells. This is in good agreement with the findings reported for non-sensitized rats (BRAY & ARSDEL 1961).

After incubation of the mast-cell suspension with antigen (horse serum) a release of intracellular histamine of about 30–50 % of the total amount in the sample was found. This agrees with the findings of KELLER (1962a). If the rats were treated with antirheumatic agents towards the end of the sensitization period, steroid as well as non-steroid substances gave rise to a significant inhibition of histamine release, to an extent depending upon the dosage used. As the administration of these substances does not alter the concentration of mast cells, the total histamine content of the specimen or the amount of histamine released by the manipulation, the reduced histamine release must be due to a decreased liberation of intracellular histamine from the mast cells in the specimen after the antigenantibody reaction.

Administration of any of the glucocorticoids studied led to a significant inhibition of histamine release, and the calculated ED50 doses (corresponding to a 50 % inhibition of histamine release) amounted to 75, 65, 4 and 2 mg/kg for hydrocortisone, fluorometholone, methyl prednisolone and betamethasone, respectively. As the clinical potency ratio of these substances is 1 : 2 : 5 : 30 (WEST 1958, BOLAND 1962) it may be seen that

the relative activities of these substances correspond fairly well with their clinical anti-inflammatory potency

Phenylbutazone inhibited histamine release significantly at a dosage of 200 mg/kg, but oxiphenbutazone and monophenylbutazone showed no change in histamine release. Salicylate, acetylsalicylic acid amidopyrine and chloroquine had no effect, whereas sodium aurothiosulphate at a low dosage gave rise to significant inhibition. It has been demonstrated that mepyramine, and also chlorprothixene and amitriptyline, show an effect in several anti-inflammatory tests. The two last mentioned agents, at a dosage of 25 mg/kg, inhibit dextran and carrageenin-induced oedema as well as granuloma formation induced by subcutaneous implantation of carrageenin pellets into rats. Mepyramine, chlorprothixene and amitriptyline have no effect on histamine release Among the substances investigated only potent antirheumatic agents were able to inhibit histamine release. The method accordingly appears to be applicable for evaluating antirheumatic substances.

The *in vitro* experiments show no inhibition of histamine release on addition of corticoids to the mast-cell suspension before incubation with antigen On the other hand, the release was inhibited by phenylbutazone at concentrations of 100 and 1000 µg/ml and by sodium salicylate at concentrations of 1000 and 10,000 µg/ml The results are in complete conformity with the alterations in histamine release obtained by *in vitro* experiments on perfused lungs from sensitized guinea pigs (TRETHEWIE 1957 & 1958) The effects of sodium-γ resorcylate and amidopyrine correspond to that of sodium salicylate. The most marked effect was obtained with chlorprothixene and amitriptyline, which inhibited histamine release at a concentration of 10 µg/ml. According to the results, there is no parallelism between the effects of the substances in the *in vitro* and in the *in vivo* tests.

Summary

A method has been developed for determining the ability of drugs to inhibit the release of histamine after an antigen-antibody reaction in a suspension of peritoneal mast cells from sensitized rats.

If the rats are treated with antirheumatic agents towards the end of the sensitization period, exposure of the removed suspension of mast cells to antigen (horse serum) will result in the liberation of a lower percentage of the total histamine content than in a control group Only clinically potent antirheumatic agents, corticosteroids as well as non-steroids, showed a significant inhibition of histamine release. This inhibition is due to a decreased liberation of intracellular histamine from the

mast cells after the antigen-antibody reaction. The relative potencies of the glucocorticosteroids tested corresponded fairly well with their clinical anti-inflammatory potencies

On direct addition of antirheumatic agents to the mast-cell suspension before addition of horse serum, no correlation was found between the clinical potency of a substance and its ability to inhibit the histamine release.

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Hepatotoxicity of Inhaled Trichloroethylene and Tetrachloroethylene. Long-term Exposure

By

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Comparative investigations into the hepatotoxicity of in particular the solvents trichloroethylene and tetrachloroethylene to mice after single exposures were reported in an earlier paper (KYLIN *et al.*, 1963) The hepatotoxic effect was estimated by histological examination together with determination of extractable liver fat and of serum ornithine carbamoyl transferase (S–OCT) After 240 minutes exposure of the animals, trichloroethylene produced only slight fatty degeneration at a concentration as high as 3,200 ppm, whereas tetrachloroethylene caused moderate fatty degeneration at 200 ppm. With neither solvent was liver cell necrosis observed. In terms of fatty degeneration the hepatotoxic effects of tri- and tetrachloroethylene were approximately as 1 10.

The experimental study reported here was designed to elucidate the hepatotoxic effects of trichloroethylene and tetrachloroethylene over a period of weeks to mice receiving repeated exposure.

Material and Methods

Solvents. Trichloroethylene (<0.2 % impurities). Tetrachloroethylene (<0.5 % trichloroethylene and <0.5 % other impurities).

Animals. Two hundred and forty female albino mice of one strain mean weight 19 grams. They received the standard diet previously described (KYLIN *et al.* 1963) with water *ad libitum*.

Exposure The exposure equipment has already been described (KYLIN *et al.* 1963). Exposure was for four hours daily six days a week, over periods of one, two and eight weeks, twenty mice being exposed in each of these subgroups.

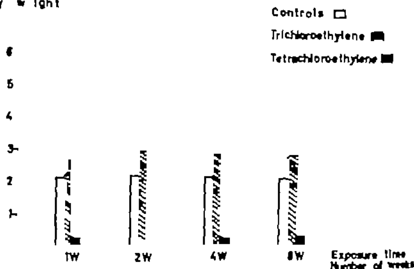


Fig. 2. Mean amounts of extractable fat in livers of controls and of mice from the groups exposed to 1 600 ppm trichloroethylene or 200 ppm tetrachloroethylene, four hours daily for six days week.

came from livers that contained only small amounts of extractable fat. A plausible explanation of this discrepancy is that occasional preparations were not representative of the organ as a whole, for in some sections the examiner noted heterogeneity of fat infiltration, and this in turn impeded evaluation of the section in its entirety

Discussion

In the previous investigation (KYLIN *et al* 1963) the hepatotoxic effect on mice of a single exposure was approximately ten times greater for tetra than for trichloroethylene. A high incidence of moderate fatty infiltration was observed after an exposure time of only four hours with 200 ppm tetrachloroethylene, but only a low incidence with 3,200 ppm trichloroethylene The present studies, in which mice were exposed at regular intervals for periods of up to eight weeks, lend further emphasis to the difference in hepatotoxicity of the two solvents. With trichloroethylene the fatty degeneration was only slight at a concentration as high as 1 600 ppm, whereas with tetrachloroethylene it was massive from as little as 200 ppm. Even the development of the morphological changes during the observation period pointed to an essential difference in hepatotoxicity of the two substances. The histologically observed tendency towards regression of the liver lesions with increasing duration of exposure

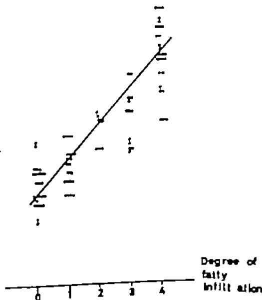


Fig. 1. Correlation between histologically evaluated degree of fatty degeneration and amount of extractable fat in livers of mice exposed to trichloroethylene or tetrachloroethylene.

to trichloroethylene may perhaps be interpreted as an enhancement of tolerance to that solvent. With tetrachloroethylene, on the contrary, the morphological changes tended to progress with longer exposure times.

Worthy of note is, further, the close agreement between the histologically evaluated degree of fatty degeneration and the amount of liver fat determined by extraction. Here the correlation coefficient was +0.74. For quantitative estimation of fatty degeneration, therefore, histological evaluation may frequently suffice, particularly if several sections are taken from different part of the liver.

REES & SHOTLANDER (1963) recently studied the accumulation of liver fat on administration of certain toxic compounds, including carbon tetrachloride. Their findings suggest that the increment is due to ... synthesis of the protein component of the lipoprotein – the form in

triglycerides are released from the liver Pronounced fatty de the liver is usually accompanied by necrosis of liver cells. H SPECTOR (1961) demonstrated, however that fatty cell necrosis are two independent processes, either of which appropriate agents, be produced separately

Thus the hepatotoxic effect of tetrachloroethylene may it consist in an inhibition of lipoprotein synthesis, leaving the *structure largely intact. Consistent with such a mechanism is* tion of KYLIN *et al* (1963) that even massive exposure to lene caused no elevation of serum transaminase. Although fatty tion does not substantially of itself alter liver function, morphosis" of long duration, clinical or experimental, is capable of leading to liver cirrhosis (POPPER & SCHAFFNER 57 1961) However none of our mice, despite the pronounced fatty tion, developed cirrhosis during eight weeks exposure to ethylene This period, constituting approximately 7 / of the average life span, was possibly too short for the development of lesions. With necrotizing agents such as carbon tetrachloride eg is, none the less, sufficient for the production of cirrhosis in animals (ADAMS *et al* 1952)

In single exposures of human subjects to threshold for neurological manifestations is approximately (STEWART *et al* 1961) This appears to have constituted the norm most widely accepted hygienic threshold of 100 ppm, the average tration for a normal workday a condition "under which it is that nearly all workers may be repeatedly exposed day after day adverse effect" (AMERICAN CONFERENCE OF GOVERNMENTAL I. HYGIENISTS 1964)

Experimental studies with tetrachloroethylene on human subj disclosed no appreciable liver damage (STEWART *et al* 1961 1963) However fairly substantial fatty degeneration may devel showing pathological values in conventional hepatic function histological investigation that might definitively resolve this has been reported. COLER & ROSSMILLER (1953) studied a group workers employed at a dry-cleaning establishment where ethylene was used. They noted pathological liver findings in uals, one of whom also had cirrhosis.

Pending further investigations on humans exposed to this evaluation of the hygienic threshold must be based principally results of animal experiments

Our studies reported here show that exposure of mice to 200 p chloroethylene for four hours a day – equivalent to the hygienic

of 100 ppm for eight hours a day – gives rise to pronounced fatty degeneration of the liver. In view of this finding, the acceptability of 100 ppm as a hygienic threshold is questionable; rather does it seem advisable to reduce the permissible level to 30 ppm, the threshold that, for other reasons, is recommended in Scandinavia.

Summary

The hepatotoxicity of trichloroethylene and tetrachloroethylene was studied in mice submitted to long-term exposure by inhalation. Duration of exposure was four hours daily for six days a week over periods of one, two, four and eight weeks. Effects on the liver were evaluated by histological examination and determination of extractable liver fat.

The most conspicuous histological change consisted of fatty degeneration. With trichloroethylene this lesion was slight even at a concentration of 1 600 ppm, but with tetrachloroethylene it was massive at as little as 200 ppm.

Neither liver cell necrosis nor cirrhosis was discernible.

On the basis of these findings the hygienic threshold for tetrachloroethylene is discussed.

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